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Comparative Hepatic Oxidative Biotransformation in Domestic Avian Species.

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COMPARATIVE HEPATIC OXIDATIVE BIOTRANSFORMATION IN DOMESTIC AVIAN SPECIES

A Dissertation

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ABSTRACT

This study characterized the oxidative biotransformation capacity of the hepatic cytochrome P-450 system of selected avian species representing the Galliformes (chicken, turkey and Japanese quail) and Anseriformes (goose, Pekin duck, Khaki Campbell duck, and Muscovy duck) orders. Two objectives were addressed; first, important biotransformation pathways were evaluated in economically major and minor food-producing avian species to provide a framework on which to base drug approval decisions in these species. Second, the potential use of the selected species as sentinel organisms utilizing cytochrome P-450 induction as a biomarker of environmental chemical exposure was evaluated. The male rat was utilized for the mammalian model and as a point of reference. Two groups of animals for each species were evaluated, a control group, which received no treatment, and a treatment group which was administered β-naphthoflavone (βNF). Investigations included the quantitation of microsomal protein, cytochrome P-450, NADH- and NADPH-cytochrome *c* reductase activity, ethoxyresorufin *O*-deethylase activity and benzo[*a*]pyrene hydroxylase activity with the development of profiles for four major metabolites.

This study found that constitutive microsomal protein levels, cytochrome P-450 content, NADPH-cytochrome *c* reductase activities and ethoxyresorufin *O*-deethylase activities were similar among the species studied while NADH-cytochrome *c* reductase activities differed. In response to βNF, microsomal protein levels, cytochrome P-450

content, and ethoxyresorufin *O*-deethylase activities were increased but no change was observed in NADPH- or NADH-cytochrome *c* reductase activities. Interspecies differences were observed in the response of the hepatic microsomal system to βNF treatment.

Interspecies differences were also observed in the capacity of the avian hepatic microsomal system to hydroxylate benzo[*a*]pyrene and in the effect of βNF treatment upon metabolite production. 3-Hydroxybenzo[*a*]pyrene was the primary metabolite produced by all the species studied; interspecies and interorder differences in product distributions were observed for the 9-hydroxybenzo[*a*]pyrene, benzo[*a*]pyrene 7,8-dihydrodiol and benzo[*a*]pyrene 9,10-dihydrodiol metabolites.

1.0. INTRODUCTION

The hepatic cytochrome P-450 enzyme system functions in the metabolism of exogenous as well as endogenous compounds and, therefore, plays a critical role in controlling the biological activities of drugs, toxins and other xenobiotics. Extensive research in the biotransformation of xenobiotics has led to recognition of the biological importance of this system. The majority of cytochrome P-450 studies have been directed at the characterization of the mammalian hepatic microsomal system. Consequently, relatively little is known about the hepatic cytochrome P-450 system of avian species. Hence, further studies on the processes of xenobiotic metabolismin avian species were deemed necessary to evaluate potential risks in the use of drugs, pesticides and other chemicals in the poultry industry and for interpreting responses of domestic and wildlife avian species to environmental toxicological challenges.

The present study was conducted to characterize the oxidative capacity of the hepatic microsomal systems of Anseriformes and Galliformes birds[1] utilizing a model substrate, benzo[*a*]pyrene (B[*a*]P). The oxidative metabolic pathways of B[*a*]P have been well described in laboratory rodents and to a lesser extent in birds. Cytochrome P-450 activities in control and β-naphthoflavone (βNF) treated groups of avian species were measured and comparison of these activities were made between avian species, between avian orders and between avian species and a mammalian model, the rat.

[1]The avian species considered were the White Leghorn chicken (*Gallus gallus*), Japanese quail (*Coturnix coturnix*), turkey (*Melegris gallopavo*), Khaki Campbell duck (*Anser platyrhynchos*), Pekin duck (*Anser platyrhynchos*), Muscovy duck (*Cairina moschata*) and goose (*Anser anser*).

The resulting database provided the tools to evaluate two distinct areas of scientific interest and public concern which may be profoundly affected by the activities of the cytochrome P-450 system. These areas include drug approval processes in minor food-producing avian species; and the use of domestic avian species as environmental sentinels for ecotoxicological studies. Two objectives were addressed by this study. First, an important biotransformation pathway was evaluated in economically major and minor food-producing avian species to provide a framework on which to base drug approval decisions in these species. Second, the potential use of the selected species as sentinel organisms utilizing cytochrome P-450 induction as a biomarker of environmental chemical exposure was evaluated. The research described herein has served to initiate a database for addressing these areas for selected avian species.

1.1 Approval of Minor Use New Animal Drugs

The Food and Drug Administration (FDA) has the responsibility of ensuring that animal drugs are effective and safe for their intended use and that food products derived from medicated animals are safe for human consumption. This responsibility stems from the 1968 amendment to the Federal Food, Drug and Cosmetic Act which set forth regulations governing the use of animal drugs (21 CFR 500-599). These amendments specifically address the safe and effective use of animal drugs and the prevention of unsafe drug residues in products intended for human consumption (Food and Drug Administration, 1992). Minor use new animal drugs are defined by the FDA as drugs "(1) used in minor species of animals or (2) used in animal species for

the control of a disease that occurs infrequently and in limited geographic areas." (Food and Drug Administration, 1979; Food and Drug Administration, 1983; 21 CFR 514.1). For purposes of drug approval, the FDA defines minor species as "animals other than cattle, horses, swine, chickens, turkeys, dogs, and cats" (Food and Drug Administration, 1979; Food and Drug Administration, 1983; 21 CFR 514.1). Due to the economic limitations of the minor species industry, no drugs have received approval for use in control of disease in minor avian species. Lack of treatment in diseased flocks may result in injury or death of affected individuals and may potentially result in a significant loss in production, and may eventually present health hazards to other species or to humans whether by direct or indirect contact, or by serving as a source of unwholesome food products for human consumers (Food and Drug Administration, 1983). Of equal concern to regulatory agencies is that use of non-approved drugs in minor species may result in unexpected or unusual drug metabolism with bioaccumulation of drug or metabolites and possible development of organisms resistant to the compound and its marketed analogs (Food and Drug Administration, 1983).

In recognition of these issues and in an attempt to encourage the submission of applications for needed minor use drugs, the FDA proposed, in July 1979, to amend the animal drug regulations (21 CRF 514.1) to provide for the use of supporting safety and effectiveness data in the approval of new animal drug applications. Concurrent to the issuance of this proposal, guidelines specifying the types of data needed to meet

the requirements of animal safety and effectiveness, as set forth in the Animal Drug Act, were issued by the FDA. These guidelines included specific requirements for studies of drug applications in domestic ducks and game birds (Food and Drug Administration, 1979; Food and Drug Administration, 1986; Food and Drug Administration, 1986a). In January 1983, the final ruling on new animal drug applications was issued allowing sponsors, where appropriate, "to use data supporting an approved major use of a drug to support a new animal drug application for a minor use of the same drug" (Food and Drug Administration, 1983). In general, these regulations require prior approval of the drug in a major species in conjunction with supporting metabolic and residue data in the minor species (Juskevich, 1987). To further promote drug approval in minor species, the Food and Drug Administration Center for Veterinary Medicine (FDA/CVM) is exploring the use of alternative studies to satisfy the data requirements set forth by the animal drug regulations (Juskevich, 1987).

In support of this objective, the FDA/CVM sponsored an extramural drug research program for minor species (Dalvi, 1988; Juskevich, 1987; Teske, 1987; Short *et al.*, 1988). The objectives of this program were to "investigate the comparative physiology, metabolism, pharmacology, and toxicology of major and minor animal species and to study the applications and limitations of pharmacokinetic parameters as a basis for the extrapolation of data from approved major use drugs to minor use drugs for fish, game birds, goats, rabbits, and similar food animals" (Teske, 1987).

A portion of the FDA/CVM program was implemented as a consortium between Louisiana State University, Auburn University, University of Illinois, and Mississippi State University (Teske, 1987).

As part of the program, a study was conducted on the comparative metabolism and disposition of fenbendazole in minor species (Short, *et al.*, 1988). One phase of this project included a comparative study of the metabolism of fenbendazole by the hepatic microsomal enzyme system of the chicken, turkey, and duck. In summary, dramatic differences in the rate of ρ-hydroxylation of fenbendazole were observed among these avian species. Furthermore, the results showed that for the avian subset studied, the differences in oxidative metabolism were often greater between major species within an order (chicken and turkey) than between a minor species (duck) and a member of the major species group of another order. Studies utilizing model substrates were conducted to compare a variety of other microsomal activities in these avian species and it was found that for the various microsomal activities evaluated, the metabolic capacity of the duck was frequently different from that of the chicken and turkey. Taken together, these observations necessitated further studies in which certain metabolic activities of the avian Galliformes and Anseriformes orders were compared (Short *et al.*, 1988 and Short, *et al.*, 1988a).

In order to address the need for further information on comparative oxidative metabolism in minor and major avian species, the research project described in this

dissertation was undertaken. A major objective of this study was to characterize the aryl hydrocarbon hydroxylase (AHH) activity in Galliformes and Anseriformes birds utilizing the classical substrate model, benzo[*a*]pyrene. Characterization of a specific mixed function monooxygenase (MFO) activity, *e.g.* aryl hydrocarbon hydroxylation, has provided data for the estimation of the activity of this system and for the relative rate of oxidation of structurally similar, untested compounds in the represented avian species (Walker, 1978). Therefore, a major objective of this research was to provide information on the cytochrome P-450-dependent metabolic capacity of seven avian species for a given class of xenobiotics and to characterize the differences in metabolism of model compounds between the species. This objective was intended to develop tools to support interpretation of data submitted for the purpose of obtaining approval of minor use avian drugs.

1.2 Avian Species as Sentinels in Ecotoxicological Studies

1.2.1 Biological Markers

Chemical contamination of the environment has become a major public concern throughout the last half century (Basscietto, *et al.*, 1990; Carson, 1962; De Witt, 1955; Harris, *et al.*, 1990; Keith, 1966; Mendelssohn, 1962; Ratcliff, 1967; Peakall, 1967; Stickel, 1946). In recognition of this concern, the identification, characterization, and remediation of hazardous waste sites has become a top priority of the U.S. Environmental Protection Agency (EPA), which has a major objective of protecting human health and the environment. The regulations governing these

activities, the Comprehensive Environmental Response Compensation and Liability Act (CERCLA), as amended by the Superfund Amendment and Reauthorization Act (SARA), stipulate that the remedial actions implemented at a hazardous waste site achieve protection of terrestrial and aquatic organisms (Environmental Protection Agency, 1989). In an effort to comply with these regulations, considerable attention has been focused on the assessment of ecological risks (Bascietto, *et al.*, 1990). This focus has lead to the realization in scientific and regulatory communities that cause and effect relationships associated with environmental chemical exposures are poorly understood and that research into environmental toxicology is of paramount importance for evaluating impacts to ecological systems and for achieving compliance with environmental regulations.

Utilization of biological markers in plants and animals (sentinel species) has been explored as an innovative approach for assessing environmental quality and chemical exposure (Ahokas, *et al.*, 1976; Beardsley, *et al.*, 1978; Bend, *et al.*, 1977; Elangbam, *et al.*, 1989; Fossi, *et al.*, 1992; Khattab, *et al.*, 1993; Knight and Walker, 1982a; Kurelec, *et al.*, 1977; Lubet, *et al.*, 1992; McCarthy and Shugart 1990; Payne, 1976; Payne, 1977; Peakall, 1967; Peakall *et al.*, 1987; Rattner *et al.*, 1989; Sandhu and Lower, 1989; Talmage and Walton, 1991; Walker, 1992). A sentinel species could be any domestic or wild animal, plant or microorganism that can serve as an indicator of chemical exposure for the purpose of assessing the potential impacts of pollution on environmental and human health (EPA, 1991). A sentinel species may

be a organism indigenous to the area of concern or it may be a "control" organism confined at the site for the assessment of *in-situ* exposures (DiGiulio, 1989; Sandhu and Lower, 1989; Shugart, *et al.* 1992) . The use of sentinel species for this purpose is referred to as biological monitoring or biomonitoring (EPA 1991). The term biological marker, or biomarker, refers to the measurement of a physiological or biochemical parameter in individual organisms that serves as a sensitive indicator of environmental chemical exposure and sublethal stress (Baker, 1989). The use of biomarkers in the evaluation of environmental contamination presents a unique opportunity for environmental managers to obtain information concerning potential ecological impacts that is not available from the chemical analyses of affected environmental media and/or tissues of exposed organisms routinely conducted at hazardous waste sites. Biomarker data can provide a measure of environmental chemical exposure as well as a measure of the magnitude of the organism's response to the exposure (McCarthy and Shugart 1990; Shugart, *et al.*, 1992). Biomarkers can validate the biological significance of chemical exposure on a site-specific basis by providing evidence that the chemical(s) was absorbed by the organism, distributed within the tissues, and that a physiological or biochemical response was elicited at a critical target (McCarthy and Shugart 1990). This type of data, in conjunction with laboratory studies, can aid in the elucidation of dose-response relationships for environmental chemical exposure in wildlife species (Baker, 1989). Furthermore, biomarkers can provide a biologically relevant indication of the cumulative effect of toxicological and pharmacological interactions associated with exposure to chemical

mixtures. Biomarkers used in conjunction with site-characterization data can provide an indication of bioavailability. These can also provide evidence of exposure to constituents that are readily metabolized and excreted, and therefore do not bioaccumulate, such as organophosphates and polycyclic aromatic hydrocarbons (PAHs) (Baker, 1989; Khattab, *et al.*, 1993; McCarthy and Shugart 1990; Sandhu and Lower, 1989; Shugart, *et al.* 1992; Walker, 1992). Biomarker data can also serve as an indicator that basic physiological or biochemical responses have been disrupted at defined concentrations of environmental chemicals which in turn can provide valuable input to the assessment of potential human health hazards associated with a hazardous waste site (Baker, 1989; McCarthy and Shugart 1990; Sandhu and Lower, 1989). Based on these considerations, it is apparent that biological monitoring is an informative and logical complement to routine sampling of contaminated media for the assessment of ecological impacts resulting from chemical releases to the environment.

1.2.2 Mixed-Function Monooxygenases as Biological Markers

Many common and toxicologically significant contaminants, such as PAHs, polychlorinated biphenyls, polybrominated biphenyls, dioxins, petroleum hydrocarbons, organochlorine pesticides, and organophosphorus insecticides, are known inducers of the MFO system (refer to Section 2.1.3 for a discussion on induction of the MFO system). Based on this, induction of the MFO system has been examined for its potential use as a biomarker of environmental exposure to these types of constituents (Bend, *et al.*, 1977; DiGiulio, 1989; Kleinow, *et al.*, 1987; Knight and

Walker, 1982a; Jimenez, *et al.*, 1990; Kurelec, *et al.*, 1977; Payne, 1976; Payne, 1977; Payne, J.F., 1987; Rattner, *et al.*, 1989; Shugart, *et al.*, 1992; Walker and Ronis, 1989). In addition, there has been considerable interest in the critical role of the MFO system in xenobiotic detoxification and the toxicological implications associated with induction of this system (DiGiulio, 1989; Kleinow, *et al.*, 1987; Hutson, *et al.*, 1991; Rattner, *et al.*, 1989; Payne, J.F., 1987; Peakall, 1967; Sell, *et al.*, 1971; Walker, 1991; Walker, *et al.*, 1991; Varanasi, *et al.*, 1987). One area of interest that has been identified is the potential for increased xenobiotic activation and the augmentation of toxicity in organisms subjected to chronic environmental chemical exposures. Other concerns include the induction of tumors and reproductive impairment due to imbalances in the synthesis and metabolism of endogenous steroids. There has also been considerable interest in the potential for exposed organisms to develop an increased capacity for xenobiotic detoxification with the subsequent development of tolerance.

The use of MFO induction as an indicator of environmental chemical exposure offers a number of advantages in biological monitoring programs (Baker, 1989). Induction of the MFO system is usually detectable in exposed organisms prior to the onset of more serious pathologies. Therefore this response can be used as an early warning signal that basic biological processes have been altered as a result of environmental chemical exposure. And, unlike other physiological or biochemical responses to chemical stress, MFO induction is indicative of a detoxification response (Payne, *et*

al., 1987). Mixed-function oxidase studies can be conducted under both laboratory and field conditions, thus providing an important linkage between traditional toxicity testing and effects observed under field conditions (Baker, 1989). For example, dose-response relationships established for MFO induction under laboratory conditions provide important data for making decisions concerning the "acceptability" or "non-acceptability" of chemical concentrations detected in the environment. Mixed-function oxidase measurements made in the field provide an important index of bioavailability and biological response for actual exposure conditions (DiGiulio, 1989). And, since MFO induction is a biochemical response that crosses taxonomic lines, MFO data obtained in sentinel species can provide inferences for potential human health effects (Baker, 1989; DiGiulio, 1989).

Characterization of the mixed-function oxygenase system provides information on the constitutive and inducible properties of the xenobiotic-metabolizing enzymes of organisms that may be exposed to chemicals released to the environment. Studies of this nature can serve to identify species which may be more, or perhaps less, susceptible to environmental pollution due to the presence, or absence, of certain constitutive MFO enzymes or due to the inducibility, or lack of inducibility, of specific MFO enzymes (Walker, 1978). For example, the increased susceptibility of certain avian species to environmental contamination has been linked to species-specific characteristics of the hepatic MFO system (Knight and Walker, 1982a; Peakall, 1967; Knight, *et al.*, 1981; Walker, *et al.* 1987; Walker, *et al.*, 1991). The

detoxification of environmental contaminants, particularly pesticides, by food-producing avian species such as the chicken, turkey, goose and duck is also an important concern for the protection of human health (Walker and Ronis, 1989).

Because MFO induction responds rapidly to changes in environmental quality, it can serve as an early and sensitive warning system for the detection of potentially significant ecological effects due to environmental contamination. Biological monitoring of the MFO system of ecological species can serve as a practical and economical tool for environmental managers faced with real or perceived concerns regarding chemicals present at, or migrating from, a hazardous waste site (Payne, *et al.*, 1987). Mixed-function oxidase biomarkers can also be used as short-term indicators of changes in exposure conditions associated with changes in environmental quality and thus can serve to delineate boundary limits for point sources of contamination and provide a practical method to assess improvements in environmental quality associated with remedial efforts (Payne, *et al.*, 1987 and Harris, *et al.*, 1990).

1.2.3 Historical Perspective on the Use of Mixed-Function Monooxygenases as Biological Markers

Induction of the MFO system has been validated as a biomarker of environmental chemical exposure in a number of wildlife species (Payne, *et al.*, 1987; Lubet, *et al.*, 1990). The majority of work in the field of MFO biomarkers has focused on aquatic invertebrates and fish. Research efforts have successfully demonstrated that a variety

of organic chemicals, when released into an aquatic system, result in MFO induction and that the response is of such sensitivity that it can be used to assess water quality over broad geographic regions (Kleinow, *et al.*, 1987; Payne, J.F., 1987; Rattner, *et al.*, 1989). However, data on the applicability of MFO induction to monitor exposure in other wildlife species, such as mammals, birds, reptiles, and amphibians is very limited (Rattner, *et al.*, 1989). Interest in the MFO activity of these groups has increased in recent times due to the continuing concern over the environmental and biological fate of lipophilic contaminants released to the environment (Walker and Ronis, 1989). This interest has lead to the characterization of baseline MFO activity in a variety of avian species (Fossi, *et al.*, 1986; Peakall, *et al.*, 1987; Knight and Walker, 1982; Knight and Walker 1982a; Walker, *et al.*, 1991). In addition, a number of environmentally important chemicals have been demonstrated to induce the hepatic mixed-function oxidase system of avian species (Pan and Fouts, 1978). This finding has lead researchers to recognize the applicability of induction of the avian monooxygenase system as a biochemical indicator of environmental chemical exposure (Ronis and Walker, 1989). Available data on the effects of environmental contamination on avian species suggest that the measurement of MFO activity in embryos and hatchlings may be useful as a biomonitoring tool (Rattner, *et al.*, 1989; Hoffman, *et al.*, 1990). Data generated to date with adult birds indicate that there is high variability in MFO activity among individuals and therefore, the relationship between exposure and induction is much less predictable (Buckpitt and Boyd, 1982; Fossi, *et al.*, 1990; Peakall, *et al.*, 1987; Rattner, *et al.*, 1989). Consequently,

researchers have concluded that a more detailed characterization of the avian MFO system, and the factors that influence it, is needed before MFO responses can be applied as a routine biomarker of environmental contamination (Fossi, *et al.*, 1990; Jimenez, et al., 1990; Knight, *et al.*, 1981; Peakall, *et al.*, 1987; Rattner, *et al.*, 1989; Ronis and Walker, 1985).

Substrates that have been used to characterize induction of the cytochrome P-450 system associated with chemical exposure include cytochrome *c* (as a measure of NADPH cytochrome P-450 reductase activity), benzo[*a*]pyrene, ethoxyresorufin, pentoxyresorufin, benzyloxyresorufin, ethoxycoumarin, benzphetamine, aldrin, aniline, aminopyrine, ethylmorphine, and *p*-nitroanisole (Lubet, *et al.*, 1990; Payne, *et al.*, 1987; Peakall, *et al.*, 1986; Rattner, *et al.*, 1989; Simmons and McKee, 1992; Walker, *et al.*, 1991). The efficacy of using AHH and 7-ethoxyresorufin-*O*-deethylase (EROD) activities as indicators of exposure to 3-methylchloanthrene (3MC)-type environmental chemical inducers has been established by a number of investigators for a variety of wildlife species including birds (Lubet, *et al.*, 1990; Simmons and McKee, 1992; Payne, 1976; Rattner, *et al.*, 1989; Mineau, *et al.*, 1984; Gilbertson and Fox, 1977; Ellenton, *et al.*, 1985; Jewell, et al., 1989; Kleinow, *et al.*, 1987; Knight and Walker, 1982; Lubet, 1992; Hoffman, *et al.*, 1987; Martin, *et al.*, 1987; Walker, 1991). Due to the degree of variation in substrate specificity of phenobarbital (PB)-inducible forms of cytochrome P-450, pentoxyresorufin and benzoxyresorufin dealkylase activities have been recommended as sensitive biomarkers

of P-450 induction for the detection of exposure to phenobarbital-type environmental inducers in mammals (Lubet, *et al.*, 1990; Simmons and McKee, 1992). However, preliminary studies indicate that the chicken has very low basal levels of pentoxyresorufin depentylase and that this activity is not inducible by phenobarbital in this species (Lorr and Bloom, 1987). Therefore, the use of this activity for biomonitoring purposes in avian and other nonmammalian species requires further study. Hepatic MFO activities are most commonly utilized for biomonitoring purposes but some work has been done to characterize induction of MFO systems present in other tissues such as the skin, kidney, and duodenum (Lubet, *et al.*, 1990; Knight and Walker, 1982).

The development and interpretation of biomarker responses and the validation and application of biomarker-based monitoring is a relatively new field (Shugart, *et al.*, 1992) and the association between MFO induction and environmental contamination has not yet been unequivocally demonstrated (Payne, J.F., 1987; Rattner, *et al.*, 1989.) Nevertheless, based on the demonstrated success of using MFO induction as a biomarker in aquatic species and the encouraging data generated in other wildlife species, further study on the use of MFO activity as a biomonitoring tool is warranted (Rattner, *et al.*, 1989). Numerous directions for future research in wildlife MFO have been proposed by investigators. Recommendations relevant to this study include: characterization of constitutive and inducible cytochrome P-450 isozymes in avian species (Rattner, *et al.*, 1989); evaluation of the inducibility of the hepatic cytochrome

P-450 system in avian species (Rattner, *et al.*, 1989); and investigations of the metabolism of environmentally relevant substrates, such as benzo[*a*]pyrene including the quantitation of individual metabolites by HPLC analysis (Payne, *et al.*, 1987).

The second specific objective of this study was, therefore, to characterize AHH and EROD activities in control and βNF-treated Anseriformes and Galliformes birds to evaluate the use of cytochrome P-450 induction as a biomarker of environmental chemical exposure in avian species.

2.0 LITERATURE REVIEW

2.1 Cytochrome P-450-Dependent Mixed Function Monooxygenase System

The cytochrome P-450 dependent mixed function monooxygenases were first identified in 1958 as a reduced pigment characterized by a maximum absorption of light (Soret band) at 450 nm when bound to carbon monoxide (Klingenberg, 1958; Garfinkle, 1958). In 1961, Omura and Sato provided evidence that this pigment was a new P-450 cytochrome of liver microsomes and that the unique spectral properties of this hemoprotein were lost when dissociated from the microsomal membrane (Omura and Sato, 1962; Omura and Sato, 1964, Omura and Sato, 1964a). In 1965, researchers identified the P-450 hemoprotein as having a role in the microsomal mixed function monooxygenase system (Cooper, *et al.*, 1965; Omura *et al.*, 1965). Since that time, the cytochrome P-450 system has been studied intensely leading to the recognized importance of the physiological and detoxification roles this system plays within the biological system (Ioannides, *et al.*, 1984). In the physiological role, the MFO system functions in the biosynthesis and metabolism of endogenous substances such as steroid hormones, bile acids, cholesterol, fatty acids, and vitamin D (Coon et al., 1992; Gonzalez, 1989; Kupfer, 1980; Parke, 1980; Waterman, et al., 1986). In the detoxification role, the cytochrome P-450 monooxygenases function in the metabolism of foreign chemicals, thus providing a protective mechanism against the accumulation and harmful effects of these chemicals (Ioannides, *et al.*, 1984).

In general, chemicals that are fat-soluble are readily absorbed through the gastrointestinal tract, lungs and skin, but are poorly excreted. These chemicals are metabolized to more water-soluble, polar metabolites. This process serves to enhance excretion of the xenobiotics. Biotransformation refers to the metabolic conversion to which a xenobiotic is subjected following uptake by an organism. The biotransformation of xenobiotics is catalyzed by a group of enzymes located primarily in the liver. The biotransformation reactions catalyzed by these enzymes have been defined by Williams (1959) to include oxidations, reductions, hydrolyses, and syntheses. These reactions occur sequentially and the extent to which a substrate undergoes metabolism is primarily dependent on chemical structure. Many xenobiotics are metabolized by a series of reactions which include oxidation, reduction, and/or hydrolysis followed by a synthetic reaction. However, there are some compounds that are metabolized via oxidation, reduction, or hydrolysis and do not undergo a subsequent synthetic reaction. Ethanol is an example of such a compound. Other compounds, such as phenol, do not undergo oxidation, reduction or hydrolysis but rather are metabolized solely via synthesis reactions. Some chemicals may be sufficiently polar to be excreted unchanged and thereby bypass metabolism altogether (Williams, 1967). Oxidation, reduction and hydrolysis reactions serve 2 functions: (1) to introduce a functional group into the molecule to enhance the water solubility, and thus, promote excretion, and (2) to transform the molecule to a suitable substrate for synthetic or conjugation reactions. The synthesis reactions function to conjugate xenobiotics directly or those metabolites produced by

the oxidation, reduction or hydrolysis reactions with endogenous moieties to further enhance water solubility and excretion. Representative compounds or groups involved in conjugation processes include glucuronic acid, glutathione, glycine, methionine as well as miscellaneous agents such as acetyl, sulfate and thio group functionalities (Williams, 1959; Williams 1967). The cytochrome P-450-dependent mixed function monooxygenases are the major enzyme system involved in oxidation and reduction biotransformations. Other enzymes which may function in the initial metabolism of xenobiotics include the flavin-containing monooxygenases; these were historically referred to as the mixed function amine oxidases (Kimura, *et al.*, 1983; Sabourin, *et al.*, 1984; Zeigler, 1988; Zeigler and Mitchell, 1972). The mixed function amine oxidases often compete with cytochrome P-450 monooxygenases in the oxidation of amine, hydrazine, and organic sulfur compounds.

2.1.1 Properties and Characteristics of the Cytochrome P-450 Monooxygenase System

The cytochrome P-450 monooxygenase system is a group of enzymes containing an iron-protoporphyrin IX prosthetic group with molecular weights ranging from approximately 48,000 to 59,000 Daltons (Estabrook, 1984; Schlenk, *et al.*, 1993). The cytochrome P-450 system involved in the metabolism of xenobiotics consists of cytochrome P-450 and NADPH-cytochrome P-450 reductase at a molecular ratio of approximately 20 to 1, respectively (Guengerich, 1988). This system is in intimate association with the phospholipid matrix of the smooth endoplasmic reticulum (Fouts, 1961; Holtzman, *et al.*, 1968). The association of this system with the membrane

structure of the endoplasmic reticulum serves to facilitate the interaction between the cytochrome P-450 molecule and the associated cytochrome reductase. It is hypothesized that the cytochrome P-450 enzymes are integral proteins of the membrane structure while the reductase is a peripheral protein anchored to the membrane surface via a hydrophobic peptide tail (Ito and Sato, 1969). Two hypotheses of membrane protein organization have been proposed to account for the transfer of electrons from one molecule of reductase to several molecules of cytochrome P-450. Yang and coworkers proposed that the fluid nature of the membrane allows the reductase molecule to transverse the membrane surface, allowing access to multiple P-450 molecules (Chapman, 1975; Lichtenberg, *et al.*, 1983; Strobel, *et al.*, 1970; Yang *et al.*, 1975). The second hypothesis proposed describes an organized protein complex within the membrane in which flavoprotein islands are surrounded peripherally by P-450 molecules (Estabrook, *et al.*, 1971; Peterson, *et al.*, 1978). Other components of this system include cytochrome b_5 and the associated reductase, NADH-cytochrome b_5 reductase (Hildebrant and Estabrook, 1971). The cytochrome P-450 dependent monooxygenase system is concentrated predominantly in the liver. However, cytochrome P-450 enzymes are also present in all other tissues, with exception of erythrocytes and striated muscle (Guengerich, 1988).

In contrast, the cytochrome P-450 system involved in endogenous metabolism consists of cytochrome P-450 and may utilize ferredoxin (an iron-sulfur protein), adrenodoxin or cytochrome P-450 reductase as the electron donor system (Niramjan et al., 1984).

These enzymes are located primarily in the mitochondria of steroidogenic tissues such as the adrenal cortex, testis, ovary, and placenta and in tissues involved in the metabolism of vitamin D such as the liver, kidney, and intestine (Waterman *et al.*, 1986). Some endogenous P-450 isozymes, like the detoxifying P-450s, are located in the endoplasmic reticulum of these tissues. The activities of endogenous P-450s are under the regulation of tissue-specific peptide hormones (Waterman, *et al.*, 1986). Those located in the mitochondria, in general, have a high degree of catalytic specificity (Guengerich, 1988).

Cytochrome P-450 exists as a group of multiple isoforms rather than as a single enzyme form. These isoforms differ in their physicochemical properties, substrate specificity, enzyme kinetics, amino acid sequencing and immunological characteristics (Lu and West, 1980). A nomenclature system has been developed for the identification of P-450 isoforms, genes and transcripts based on evolution of the P-450 superfamily (Nebert, *et al.*, 1991). According to this system, the P-450 gene is indicated by the italicized root symbol *CYP* which denotes *cy*tochrome *P*-450. This root symbol is followed by an Arabic number which designates the P-450 family. (By definition, P-450 isoforms within a family exhibit protein sequences which are $>40\%$ identical.) Following the family designation, the P-450 subfamily is indicated by a letter. [P-450 isoforms (mammalian) within the same subfamily demonstrate $>55\%$ homology]. Lastly, an Arabic number is used to denote the individual gene within the subfamily. The same nomenclature, but nonitalicized, is used for the mRNA and gene

product (isozyme) (Nebert, *et al.*, 1991). Gene families designated 1 through 4 code for the cytochrome P-450 enzymes involved in primary xenobiotic metabolism. The cytochromes P-450 involved in steroid biosynthesis are coded for by gene families 11, 17, 19, 21 and 22 (Gonzalez, 1989).

Individual cytochrome P-450 isoforms exhibit overlapping specificity with regard to substrate and the type of oxygenation reaction catalyzed. However, for certain substrates and reactions, varying degrees of selectivity may be demonstrated. This characteristic was first identified following the treatment of animals with the PAH 3MC (Conney, 1967; Conney, *et al.*, 1956; Conney *et al.*, 1957) and later with PB (Orrenius, 1965; Orrenius and Erickson, 1966; Orrenius, *et al.*, 1965). The isoforms induced following these treatments were noted to have different substrate specificities as well as different catalytic rates for similar substrates (Alvares *et al.*, 1967; Sladek and Mannering, 1966; Sladek and Mannering, 1969; Sladek and Mannering, 1969a; Mannering, 1971; and Gnosspelius *et al.*, 1969). Following the isolation of these two hemoproteins and the correlation of specific catalytic activities with each, it was recognized that two distinct cytochrome P-450 enzymes, designated as cytochrome P-450 (induced by PB) and cytochrome P-448 (induced by 3MC), were present (Sladek and Mannering, 1966). The two most notable differences in these two families of cytochromes include differences in substrate binding sites and preference of substrate. The cytochrome P-450 isoforms, designated as CYP2B1 and CYP2B2, are inducible by PB and have a broad substrate specificity with an affinity for non-planar, bulky

molecules. Specific substrates which interact with isozymes of the CYP2B subfamily, and are frequently used in the study of P-450 activity, include benzphetamine, ethylmorphine, aldrin, aminopyrine, and hexobarbital (Hodgson, *et al.*, 1980). In contrast, the isoforms inducible by 3MC and other PAHs, designated as CYP1A1 and CYP1A2, exhibit a narrow substrate specificity and react readily with rigid, planar molecules (Lewis, *et al.*, 1986; Safe, *et al.*, 1985). Substrates metabolized by this subfamily include benzo[*a*]pyrene, ethoxyresorufin, acetanilide, and *p*-nitrophenol (Hodgson, *et al.*, 1980). However, differences in substrate specificity have been noted between the CYP1A1 and CYP1A2 isozymes. For example, both isozymes catalyze the metabolism of benzo[*a*]pyrene, but CYP1A1 has been demonstrated to be approximately 50 times more active than CYP1A2 (Ryan, *et al.*, 1980). Conversely, the CYP1A2 isozyme has been shown to be more active than CYP1A1 in the metabolism of aromatic amines (Hammons, *et al.*, 1985). Another substrate, ethoxyresorufin, is metabolized almost exclusively by the CYP1A1 isozyme (Burke and Mayer, 1975; Guengerich, *et al.*, 1982; Goldstein, *et al.*, 1982). Substrates such as aniline, chlorpromazine and zoxazolamine are metabolized by both subfamilies of cytochromes (Lu and West, 1980).

Since the identification of these two important subfamilies of isozymes, researchers have identified over 150 cytochrome P-450 isoforms present in diverse phylogenetic groups which appear to be highly conserved through evolution (Coon, *et al.*, 1992).

The cytochromes P-450 that have been characterized in the rat are presented in Table 1-1 (Nebert, *et al.*, 1991; Wrighton, 1990).

2.1.2 Functions of the Cytochrome P-450 Monooxygenase System

As previously noted, a major role of the microsomal cytochrome P-450 monooxygenase system is to biotransform fat-soluble xenobiotics to hydrophilic metabolites that are more readily excreted. In this role, the microsomal monooxygenases function specifically to catalyze the oxidation of xenobiotics through the incorporation of one atom of molecular oxygen into the molecule with the reduction of the remaining oxygen atom to water (Brodie, *et al.*, 1958; Estabrook and Werringloer, 1977; Gillette, 1971; Gillette, 1966; Hodgson and Dauterman, 1980). This oxidation process requires the following sequence of cytochrome P-450 reactions: (1) Cytochrome P-450, in its oxidized form (Fe^{+3}), reacts with the substrate to form an enzyme/substrate complex; (2) NADPH, via NADPH-cytochrome P-450 reductase, transfers an electron to the complex resulting in the reduction of the heme moiety of cytochrome P-450 (Fe^{+2}); (3) The reduced cytochrome P-450/substrate complex then combines with molecular oxygen; (4) NADPH, via NADPH-cytochrome P-450 reductase, transfers a second electron to the complex and both accepted electrons, through an unknown mechanism, are transferred to the oxygen molecule creating an unstable, highly-reactive oxygen species. It is postulated that with some forms of cytochrome P-450, and with certain substrates, cytochrome b_5 serves as a transferase

Table 1-1

Rat Cytochrome P-450 Genes and Their Products

P-450 Gene[1]	Common Name(s)[1,2]	Comments[1,2,3,4,5]
CYP1A1	c, βNF-B	PAH-inducible form; benzo[*a*]pyrene hydroxylase, ethoxyresorufin-*O*-deethylase
CYP1A2	P-448, d, HCB	Isosafrole- and PAH-inducible form
CYP2A1	a1, a, 3, UT-F	PAH-inducible form; testosterone 7α-hydroxylase
CYP2A2	a2, RLM2	
CYP2A3	a3	
CYP2B1	b, PB-4,PB-B	PB-inducible form
CYP2B2	e, PB-5, PB-D	PB-inducible form
CYP2B3	IIB3	
CYP2B8	gene IV	
CYP2C6	PB1, k, PB-C, *pTF2*, P45k	Constitutive and PB-inducible form
CYP2C6P	(pseudogene)	
CYP2C7	f, pTF1	Constitutive
CYP2C11	h, M-1, 16α, 2c, UT-A, P450h	Constitutive
CYP2C12	i, 15β, 2d, UT-1, P-450i	Constitutive
CYP2C13	+g, P450g	Constitutive
CYP2C22	Md	
CYP2C22	cl17	
CYP2D1	db1, UT-H	Debrisoquine hydroxylase
CYP2D2	db2	
CYP2D3	db3	
CYP2D4	db4	

(table continued)

Table 1-1

Rat Cytochrome P-450 Genes and Their Products

CYP2D5	db5, CMF1b	
CYP2E1	j, P450j	Ethanol-, acetone-, isoniazid, pyrazole- inducible form; nitrosodimethylamine N-demethylase
CYP2G1	olf1	
CYP3A1	pcn1, PCN-E, P-450p	PB-, macrolide antibiotic-, steroid-inducible form; erythromycin N-demethylase
CYP3A2	pcn2	PB-inducible form; sex-specific expression (male)
CYP3A9	olf2	
CYP4A1	LA_{ω}	
CYP4A2	IVA2	
CYP4A3	IVA3	
CYP4A8	PP1	
CYP4B1	form 5	
CYP7	7α	
CYP11A1	scc	
CYP11B1	11β	
CYP11B3	11β aldo 2	
CYP19	arom	
CYP27	25-hydroxylase	

[1]Nebert, *et al.*, 1991
[2]Wrighton, 1990
[3]Ryan, *et al.*, 1980
[4]Guengerich, *et al.*, 1982
[5]Goldstein, *et al.*, 1982

for the second electron equivalent. In this role, cytochrome b_5 is thought to accept an electron from NADH via NADH-cytochrome b_5 reductase and to transfer the electron to the oxygenated substrate/P-450 complex. The function of the NADH-cytochrome b_5 microsomal electron transport chain system in P-450 catalyzed reactions has not yet been fully established (Correia and Mannering, 1973; Guengerich, 1988; Hildebrant and Estabrook, 1971; Mannering, *et al.*, 1974; Oshina and Sato, 1972); (5) One atom of oxygen is then incorporated into the substrate molecule and the other is reduced to water; (6) The cytochrome P-450/oxygenated substrate complex dissociates releasing the oxidized substrate metabolite and regenerating the cytochrome P-450 molecule thus completing the cytochrome P-450 catalytic cycle (Figure 2-1). The term mixed-function monooxygenase or oxidase is frequently used to refer to the cytochrome P-450 enzymes, due to the requirement of this system for both molecular oxygen and a reducing agent (Mason, 1957; Mason, 1965).

The rate limiting step in the cytochrome P-450 catalytic cycle varies with the P-450 isoform and the substrate. Electron transfer (Davies, *et al.*, 1969), hydrogen extraction and product release have all been identified as rate-limiting steps in various cytochrome P-450 reactions (Guengerich, 1991). The specific types of oxidative reactions catalyzed by the cytochrome P-450 enzymes include aliphatic and aromatic hydroxylation, epoxidation, *N*-, *O*-, *S*-dealkylation, deamination, sulfoxidation, desulfuration, *N*-hydroxylation, and oxidative dehalogenation (Williams, 1959;

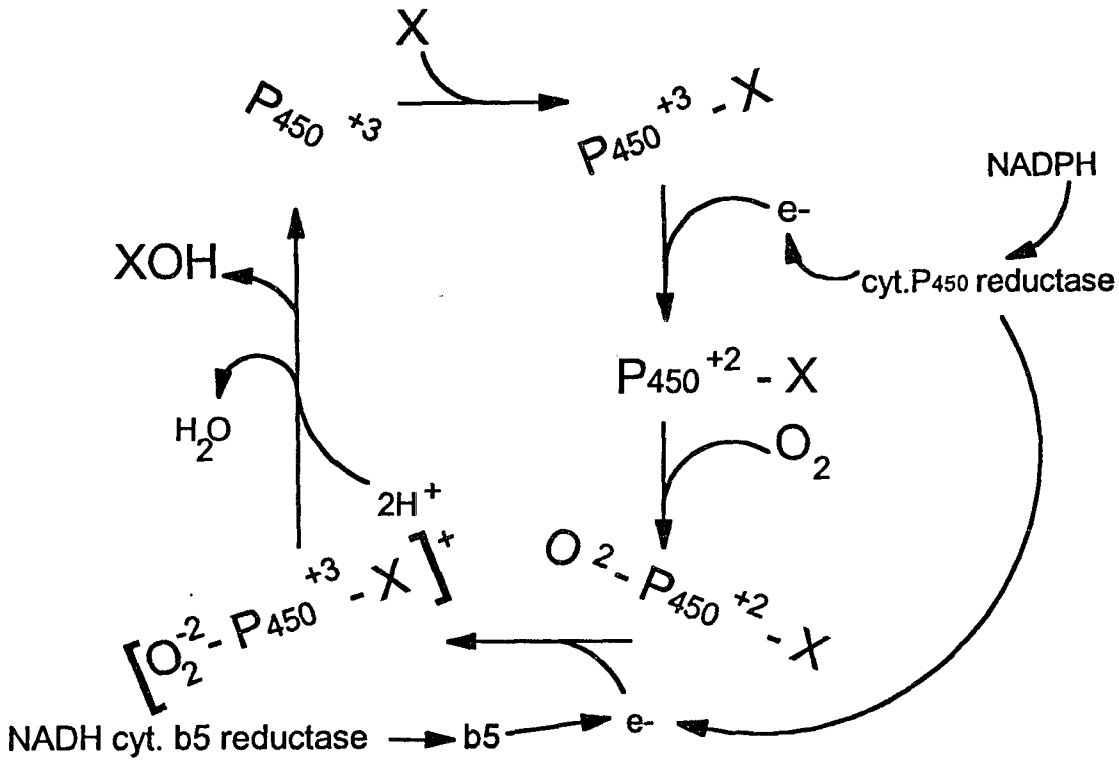

Figure 2-1 Cytochrome P-450 catalytic cycle.

Williams, 1967). To a lesser extent, the cytochrome P-450 monooxygenases also catalyze reduction reactions. These reactions include azo reduction, aromatic nitro reduction, and reductive dehalogenation (Williams, 1959). Depending on the physicochemical characteristics of the substrate, it has been postulated that all of these reactions could be catalyzed by a single P-450 isozyme (Guengerich, 1988). The biotransformation process, in general, yields products with decreased activity and, therefore, functions in the role of detoxification. However, with some compounds, biotransformation reactions result in the formation of intermediate or final products that exhibit greater reactivity than the parent compound, thereby serving as a bioactivation mechanism (Hodgson and Dauterman, 1980). For example, the oxygenation of PAHs in conformationally hindered positions, such as the bay region, results in the formation of reactive epoxide intermediates (Brookes and Lawley, 1964; Gelboin, 1969; Grover and Sims, 1968; Miller, 1951; Boyland, 1950; Conney, *et al.*, 1957; Sims, 1975; Sims, *et al.*, 1974; Parke and Ioannides, 1984; Ioannides and Parke, 1987). These electrophilic metabolites react with critical nucleophiles, such as DNA, which may lead to various pathological processes. Another example is the conversion of parathion, a relatively nontoxic organophosphate pesticide, to paraoxon which is a potent inhibitor of cholinesterase activity (Davison, 1954; Davison, 1955). The bioactivation and/or detoxification of a xenobiotic is dependent on the catalytic selectivity of the individual cytochrome P-450 isoforms present. Therefore, the balance between the overall activation and detoxification of a xenobiotic is influenced by the P-450 isoforms present and the partitioning of the xenobiotic between the

metabolic pathways catalyzed by the different isoforms (Guengerich and Liebler, 1985). Other factors which influence this balance include genetics, age, nutrition, and the selective induction of cytochrome P-450 isozymes following chemical exposure (Parke and Ioannides, 1984). For example, an increase in the activity of the CYP2A isozyme family (as with induction following PB administration) will result in an increase in the activation of some compounds such as 2-naphthylamine and cyclophosphamide while an increase in detoxification will occur with other xenobiotics such as aflatoxin and *N*-2-fluorenylacetamide (Hodgson, *et al.*, 1980).

2.1.3 Factors Affecting the Cytochrome P-450 Monooxygenase System and Biotransformation

One of the most profound factors influencing the biotransformation of xenobiotics is the enhancement of cytochrome P-450 activity following exposure to certain chemicals. This process is referred to as induction and results from an increase in the rate of synthesis of cytochrome P-450 enzymes (Conney, 1967). Hundreds of chemicals have been demonstrated to induce the cytochrome P-450 system, but the most widely studied inducing agents are PAH and PB. The two major families of inducible cytochromes associated with these agents include CYP1A (PAH-inducible isoforms) and CYP2B (PB-inducible isoforms) (Nebert, *et al*, 1991). In addition to PAHs, the *CYP*1A1 family is induced by such compounds as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Poland and Glover, 1973), βNF (Lau and Strobel, 1982), planar polyhalogenated biphenyls (Parkinson, *et al.*. 1983), aminoazobenzenes (Degawa, *et al.*, 1985), aromatic amines (Ioannides, *et al.*, 1984)

and amides (Astrom and DePierre, 1985). The *CYP*2B family is also induced by nonplanar polyhalogenated biphenyls and the pesticides 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)-ethane (DDT) and gamma chlordane (Parkinson, *et al.*, 1983). Constitutive levels of these isoforms comprise less that 5 percent of the total hepatic cytochrome P-450 population (Pickett, *et al.*, 1981; Waxman and Walsh, 1982). Following induction, their levels may increase 70-fold (Thomas, *et al.*, 1979; Thomas, *et al.*, 1981) and thereby comprise nearly 80 percent of the total cytochrome P-450 content (Ioannides and Parke, 1987). In the absence of induction, the P-450 gene subfamily *CYP*1A is not expressed in extrahepatic tissues while the *CYP*2B subfamily is, most notably in the lung and sebaceous glands (Freidberg, *et al.*, 1990). Other agents, such as Aroclors (polychlorinated biphenyl mixtures) (Parkinson, *et al.*, 1983), safrole and isosafrole, and certain drugs (phenothiazine and chlorpromazine) (Thomas, *et al.*, 1983), have been demonstrated to act as mixed-type inducers by producing a response characteristic of both PB and PAH induction (Ioannides and Parke, 1987). The onset, magnitude and duration of induction may vary with species, strain and sex of the organism, type and dose of chemical received or administered, the duration of the exposure and the tissue and substrate used to measure monooxygenase activity (Hodgson *et al.*, 1980). Not all agents capable of inducing the cytochrome P-450 monooxygenases are readily metabolized by the system (Guengerich, 1988). In addition, the induction of one family of cytochromes P-450 may result in the suppression of another family of P-450s (Vlasuk, *et al.*, 1982; Vlasuk, *et al.*, 1982a, Serabjit-Singh, *et al.*, 1983).

The mechanism of cytochrome P-450 (*CYP*1A) induction has been described for PAH-type inducing agents. Initiation of the induction process involves the binding of the inducing agent or its metabolite with a cytosolic receptor [referred to as the aromatic hydrocarbon (Ah) receptor], translocation of the receptor-ligand complex to the nucleus, reaction of the complex with specific genomic recognition sites, transcriptional activation of the *CYP*1A1 and *CYP*1A2 genes and a subsequent increase in cytoplasmic CYP1A1 and CYP1A2 mRNA and associated proteins (Poland and Glover, 1976; Poland and Knutson, 1982). This regulation process plays an important role in determining individual risk of environmentally-caused toxicities and cancer since the *CYP*1A1 gene product is thought to activate procarcinogens, such as benzo[*a*]pyrene (Heidelberger, 1975; Miller, 1970; Kapitulnik, *et al.*, 1978; Nebert and Jones, 1989; Sims and Grover, 1974). Induction of the *CYP*4 gene family by clofibrate, a hypolipidemic drug, has also been demonstrated to occur via a receptor (Lalwani, et al., 1987). For the other types of inducers, neither receptors nor molecular mechanisms governing the induction response have been identified. However, it has been demonstrated that the *CYP*2 and *CYP*3 gene families, induced by PB (Atchison and Adesnik, 1983; Hardwick *et al.*, 1983; Pike, *et al.*, 1985) and dexamethasone (Gonzalez, *et al.*, 1986; Hardwick, *et al.*, 1983), respectively, are regulated via transcriptional activation which leads to an increase in mRNA and cytochrome P-450 in the endoplasmic reticulum.

In contrast to the induction process, certain chemicals are able to inhibit cytochrome P-450 monooxygenase activity and, thus, decrease the ability of the system to metabolize xenobiotics (Conney, 1967). The inhibition of cytochrome P-450 activity is dependent on the substrate, type of reaction and type of isozymes present (Lu and West, 1980). For example, ellipticine inhibits B[*a*]P hydroxylation in constitutive, PAH-induced and PB-induced microsomes while 7,8-benzoflavone only inhibits this activity in PAH-induced microsomes (Weibel, *et al.*, 1971). Inhibitory mechanisms and representative agents that have been described include, but are not limited to, decreased synthesis of P-450 enzymes (3-amino-1,2,3-triazole), generalized destruction of P-450 (associated with lipid peroxidation), competitive inhibition due to two or more xenobiotics competing for P-450 active sites (benzene and toluene metabolism), suicide inhibition due to destruction of the heme moiety by a reactive metabolite (vinyl chloride, allylisopropylacetamide), coordination to the heme moiety (cyanide); destruction of the protein structure by a reactive metabolite (carbon disulfide, parathion, chloramphenicol), and reduction of P-450 content due to loss of tissue mass (DeMatteis, 1978; Halpert and Neal, 1980; Guengerich and Strickland, 1977; Oritz de Montellano and Correia, 1983; Oritz de Montellano and Mico, 1981; Sato and Nakajima, 1979).

Other important factors affecting biotransformation include species, strain, sex, age, and diet. Quantitative and qualitative differences in the cytochrome P-450 monooxygenase system and associated activities have been described for different

species and strains. These differences in the cytochrome P-450 system have been attributed, in part, to variations in isozymes, content, and response to inducers and the presence of enzyme defects or unique enzyme activities in certain species (Walker, 1980; Williams, 1967). In addition, variation in genetic constitution among individuals within a given species or strain has been identified as a source of variation in monooxygenase activity. This source of variation is expected to be considerably greater in wild species than in laboratory species (Walker, 1980). Gender differences in cytochrome P-450 monooxygenase activity have been noted in several species of laboratory animals with the most dramatic difference demonstrated in the rat. The female rat has been shown to have a 10 to 30 percent lower hepatic microsomal P-450 content than the male rat and also has been observed to have a slower rate of xenobiotic and drug metabolism. This profound sex-related difference is seen at 4 to 51 weeks of age and is thought to be due to differences in the contribution of sex-specific P-450 isoforms to the total P-450 content (Chengelis, 1988; Kato and Onoda, 1970; Waxman, *et al.*, 1985). Other sex-related differences that have been observed in the MFO of the rat include a greater NADPH-cytochrome *c* reductase activity in the male at age 12 to 78 weeks, greater NADH-cytochrome *c* reductase activity in the female at age 12 to 51 weeks and inconsistent differences in cytochrome b_5 at various ages (Chengelis, 1988). Sex-related variations in MFO have been observed in some species of birds at certain stages of the reproductive cycle. A discussion of these findings is presented in Section 2.3.1. Gender differences in cytochrome P-450 content and activity have also been demonstrated in certain fish species. For these

species, the females have generally been shown to have lower P-450 content and activity than their male counterparts (Kleinow, *et al.*, 1987). Differences in age have also been shown to affect xenobiotic biotransformation. The cytochrome P-450 monooxygenase system of mammalian species has been shown to develop during the gestational period with the onset of maturation beginning at birth (Short, *et al.*, 1976). Postnatal patterns of development have been observed to vary with the species and substrate studied. For the rat, MFO activity has been shown to peak at 3 weeks of age in the female and at 3 to 8 weeks of age in the male. Following the peak, activity levels plateau or decrease in the female and plateau in the male (Short, *et al.*, 1976). In contrast to mammals, avian species, in general, have been demonstrated to have significant MFO activity during the embryonic and neonatal periods. A discussion of the development of the avian MFO system is presented in Section 2.2.1. Age-related differences in the cytochrome P-450 content and activity have also been observed in certain fish species (Kleinow, *et al.*, 1987). Another age-related difference is seen in older animals. Older animals have a decreased capacity for biotransformation due to decreased concentrations of cytochrome P-450 and its associated reductase. Other related factors may include decreased hepatic and renal blood flow, reduced hepatic mass and decreased efficiency of excretory systems (Sipes and Gandolfi, 1991). Diet can affect biotransformation in a number of ways. Nutritional deficiencies, such as vitamin, mineral, and protein deficiencies, or periods of starvation, can decrease cytochrome P-450 monooxygenase activity by reducing basal levels of cytochrome P-450. They can also reduce the availability of high-energy cofactors required in Phase

II reactions. Furthermore, chemicals present in the diet can affect biotransformation through the induction or inhibition of certain P-450 activities (Walker, 1980; Yang, *et al.*, 1992). For example, diallyl sulfide, a natural constituent of garlic (Brady, *et al.*, 1991); phenethyl isothiocyanate, a component of cruceriferous vegetables (Ishizaki, *et al.*, 1990); and psoralens, present in edible plants such as parsley, celery, and parsnip (Tinel, *et al.*, 1987) have been demonstrated to inactivate certain cytochrome P-450 isozymes. Other dietary components such as ethanol (Koop, *et al.*, 1982) and flavinoids (Conney, 1982) have been reported to enhance cytochrome P-450-dependent activities.

2.2 Avian Hepatic Cytochrome P-450 Monooxygenase System

The avian hepatic cytochrome P-450 monooxygenase system is similar in structure and function to the mammalian MFO system. However, there are some important differences between these classes in relation to cytochrome P-450 content, activity, and regulation. In addition, there are significant qualitative and quantitative differences in the MFO systems of species within the Aves class.

2.2.1 Cytochrome P-450 Content and Activity

Mixed function monooxygenase studies have been conducted on a variety of avian species which collectively represent 10 orders: Passeriformes, Striges, Accipitres, Anseriformes, Pelecaniformes, Tubinares, Pygopodes, Columbiformes and Galliformes (Ronis and Walker, 1989; Walker and Ronis, 1989). The majority of the

work has focused on characterizing basal MFO activity of birds and lesser amounts of data are available on the induced system. The cytochrome P-450 content of avian hepatic microsomes has, on average, been shown to be approximately one-fourth that of mammalian microsomes (Walker and Ronis, 1989). It is currently speculated that this difference in P-450 content is due to differences in endogenous metabolism between the avian and mammalian classes. Within the Aves class, Passeriformes (house sparrow, black bird, African bulbul, starling) and Galliformes (domestic fowl, grey partridge, red partridge, Japanese quail) demonstrate relatively high levels of cytochrome P-450 (0.20 to 0.51 and 0.18 to 0.45 nmol P-450/mg protein, respectively) while the fish-eating Pelecaniformes (cormorant, shag) and Charadriformes (puffin, razorbill, guillemot) demonstrate relatively low P-450 levels (0.15 to 0.29 nmol P-450/mg protein) compared to other orders of birds (0.3 nmol/ mg protein on average) (Ronis and Walker, 1989; Walker and Ronis, 1989). In keeping with the relatively low levels of P-450 in birds, the monooxygenase activities reported in avian species are, in most cases, lower than those in mammalian species. Walker and Ronis (1989) reported that, in general, avian monooxygenase activities for aminopyrine, aldrin, and HCE (1,2,3,4,9,9-hexachloro-1,4,4a,5,6,7,8,8a-octahydro-exo-7,8-epoxy-1,4-methanonaphthalene) have been shown to be lower than those seen in mammals and in contrast, activity levels observed for aniline hydroxylation (ANH) are comparable to those observed in mammals while the activity levels for EROD are greater (Walker and Ronis, 1989).

Most monooxygenase activity in the bird is present in the liver but other tissues have been demonstrated to have significant MFO activity in certain species. For example, the duodenum has been shown to have relatively high MFO activity compared to the liver in the grey partridge, domestic fowl and Japanese quail (Galliformes) for certain substrates (Bartlet and Kirinya, 1976; Riviere, 1980; Riviere, *et al.*, 1983). In contrast, duodenal activity in the cormorant was relatively low (Knight and Walker, 1982). Avian species have also been shown to have significant MFO activity in the kidney (Pan and Fouts, 1978). This finding is postulated to be associated with the renal portal shunt present in birds (but not in mammals) which carries blood from the gastrointestinal tract directly to the kidneys. Renal activity in the razorbill and herring gull, in contrast to other birds, was found to be relatively low (Ronis and Walker, 1989). Buckpitt and Boyd (1982) investigated the P-450 system in the lung and kidney of the Japanese quail and the chicken and found relatively low P-450 content and oxidative activity towards the substrate 4-ipomeanol. Although MFO activity has been shown to be high in some extrahepatic tissues, the overall contribution of these MFO sources to the total MFO activity is minor due to the relatively small mass of these tissues compared to that of the liver. Nevertheless, these extrahepatic activities may play a significant role in the detoxification and/or activation of certain xenobiotics in target tissues (Ronis and Walker, 1989).

An interesting feature of the avian MFO system is the reported presence of significant activity in embryos and neonatal birds. Hepatic monooxygenase activities in the

chicken embryo have been demonstrated to be comparable to or greater than those of an adult from day 3 of incubation through hatching (Drummond, *et al.*, 1972; Hamilton, *et al.*, 1983; Powis, *et al.*, 1976). Immediately following hatching, hepatic MFO activities have been observed to reach a peak and then gradually decrease to adult levels within one week (Haug, *et al.*, 1980; Brunstrom, 1986). In addition, substantial hepatic MFO activity has been shown in the embryos of the Herring gull (Ellenton, *et al.*, 1985; Boersma, *et al.*, 1986; Peakall, *et al.*, 1986) and black-crowned night heron (Hoffman, *et al.*, 1986). This characteristic of the avian MFO system is in sharp contrast to the development of the MFO system in mammals. Mixed function monooxygenase activity in mammals is absent or very low at birth and gradually develops during the postnatal period (Short, *et al.*, 1976).

Data on temporal variations in activity in birds are very limited and are discussed in Section 2.3.1. Gender differences in MFO activity are seldom seen in avian species (Ronis and Walker, 1989). However, a few exceptions have been noted and these observations are discussed in Section 2.3.1.

2.2.2 Induction of the Avian Cytochrome P-450 System

In general, the response of the avian MFO system to inducers has been demonstrated to be similar to that of mammals. However, there are some important distinguishing characteristics between the Aves and Mammalia classes (Ronis and Walker, 1989). The avian monooxygenase system, represented by the chicken, Japanese quail, homing

pigeon, robin and Forster's tern, has been shown to be readily induced by 3MC, TCDD and other 3MC-type inducers such as βNF, PAHs, and coplanar PCBs (Brunstrom, 1990; Brunstrom, 1992; Hoffman, *et al.*, 1987; Martin, *et al.*, 1987; Husain, *et al.*, 1982; Husain, *et al.*, 1984; Ronis and Walker, 1989). The pattern of induction observed is similar to that seen in mammals, i.e., increases in P-450 content and AHH and EROD activities. Distinct differences in the induction response to 3MC-type inducers that have been seen in avian species, which are not seen in the mammalian species, include the induction of aminopyrine *N*-demethylase (APDM), ethylmorphine *N*-demethylase (EMDM), biphenyl 2-hydroxylase (Ronis and Walker, 1989) and 4-dimethylaminoantipyrine *N*-demethylase activities (Sawyer, *et al.*, 1986). In addition, 3MC-type inducers tend to produce a greater increase in microsomal protein and total P-450 content in birds than in mammals (Buynitzky, *et al.*, 1978; Ronis and Walker, 1989) and there is some evidence that MFO activity in the Japanese quail is induced to a greater extent by 3MC than in mammals (Ronis and Walker, 1989). Also, 1,2,4-trichlorobenzene has been shown to induce EROD activity in the quail and the chicken but not in the rat (Riviere, *et al.*, 1985; Miranda, *et al.*, 1984). Another interesting difference in the inducibility of the avian MFO system is the reported ability of the embryonic system to be induced by 3MC-type inducers. Mixed function monooxygenase activity of the chicken embryo has been demonstrated to be highly sensitive to induction by 3MC, βNF, TCDD, and 3,3',4,4'-tetrachlorobiphenyl (Althaus *et al.*, 1972; Brunstrom, *et al.*, 1976; Darbey, *et al.*, 1984; Darbey, *et al.*, 1985; Darbey, *et al.*, 1986; Dogra and Israels, 1987;

Hamilton, *et al.*, 1983; Poland and Glover, 1977; Topp and van Bladeren, 1986). Aryl hydrocarbon hydrolase activity has been demonstrated to be inducible in the chicken at day 5 of incubation, with inducibility reaching adult levels by day 7 and peaking at day 1 post hatch (Hamilton, *et al.*, 1983). Limited study has been conducted on the role of a high affinity cytosolic Ah receptor in the induction of the avian hepatic cytochrome P-450 system by 3MC type inducers. Sawyer and coworkers (1986) were unable to detect a receptor in embryo or 2-week-old chickens. In contrast, Denison *et al.* (1985) reported low levels of the receptor in the cytosol of chick hepatocytes and Brunstrom and Lund (1988) detected a TCDD receptor in the liver of 7-day-old chick embryos. However, in the same study, a receptor was not detected in the embryonic turkey liver (Brunstrom and Lund, 1988). Brunstrom (1992) demonstrated that coplanar PCBs and PAHs avidly bind to an Ah receptor in chicken embryos and that the most toxic compounds were the most avid Ah receptor binders. The coplanar PCB, 3,3'4,4'-tetrachlorobiphenyl, and its hydroxy metabolites, were shown by Wehler *et al.* (1990) to bind to an Ah receptor in chick embryos.

Variable responses to PB and PB-like inducers such as DDT and dieldrin, have been reported in avian species. The chicken has been shown to be readily induced by PB and the pattern of induction is similar to that of the rat (Walker and Ronis, 1989). Other species that have been demonstrated to be sensitive to PB induction include the homing pigeon, ring dove, Khaki Campbell duck and mallard duck (Husain, *et al.*,

1981; Husain, *et al.*, 1984; Patterson and Roberts, 1971; Peakall, *et al.*, 1970; Sifri, *et al.*, 1975). In contrast to these species, the Japanese quail and fish-eating birds, such as the cormorant, are reported to be refractory to induction by PB (Ronis, *et al.*, 1987; Walker and Ronis, 1989). It is also noteworthy that the chicken, although observed to be responsive to PB, was not shown to be responsive to DDT and dieldrin which are PB-type inducers (Walker and Ronis, 1989). In addition, Davison, *et al.* (1976) showed that mirex, another organochlorine compound, did not induce the MFO systems of the chicken and quail. The mallard duck, on the other hand, was readily induced by PB as well as DDT and dieldrin (Davison and Sell, 1972; Walker and Ronis, 1989). In fact, in one study, PB was shown to increase ANH activity in the Khaki Campbell duck twice as much as in the rat (Patterson and Roberts, 1971). It is interesting to note that, in another study, Buynitzky and coworkers (1978) demonstrated that induction following exposure to DDT was greater in mallard ducks infected with the duck hepatitis virus than in control ducks. Other compounds that have been demonstrated to produce PB-like patterns of induction in birds include allylisopropylacetamide (Rifkind, *et al.*, 1982) and organophosphorus pesticides (Lapadula, *et al.*, 1964).

Unlike the mammal, PB induction has been observed in birds at very early stages of development (Lorr and Bloom, 1987; Ronis and Walker, 1989; Strittmatter and Umberger, 1969; Topp and Bladeren, 1986). In addition to PB, other compounds that have produced PB-type induction in the chick embryo include the porphyria-causing

drugs 2-propyl-2-isopropylacetamide (Althaus, *et al.*, 1972) and 3,5-diethylcarbonyl-1,4-dihydrocollidine (Rifkind, 1979) and the *in vitro* inhibitors metyrapone, SKF525A, and nicotinamide (Rifkind, *et al.*, 1974; Giger and Meyer, 1982).

Compounds such as polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), and hexachlorobiphenyl (HCB), which are classified as mixed-type inducers, have also been studied in avian species. Induction has been observed with these inducers in the Japanese quail (Bunyan and Page, 1978; Carpenter, *et al.*, 1985; Riviere, *et al.*, 1985), adult chicken (Ehrich and Larsen, 1983), chick (Rinsky and Perry 1981), chick embryo (Rifkind, *et al.*, 1984; Topp and van Bladeren, 1986), nestling and adult barn owl (Rinsky and Perry 1981; Rinsky and Perry 1983) and Eider duck (Brouwer, 1991). In addition, the carcinogen 2-acetylaminofluorene was shown to produce a mixed-type induction in the chick embryo (Darby, *et al.*, 1984).

Other compounds that have been investigated for their effect upon the avian MFO monooxygenases include ergosterol biosynthesis inhibiting fungicides, glucocorticoids, ethanol, glutethimide, aflatoxin B_1, fenbendazole, and pyrethroids. Prochloraz, an ergosterol biosynthesis inhibiting fungicide, has been shown to be a potent inducer of hepatic and duodenal MFO activity in a variety of birds including the Japanese quail (Riviere, *et al.*, 1983), chicken, grey partridge (*Perdix perdix*), pheasant (*Phasianus colchicus*) (Riviere, *et al.*, 1984) and the red-legged partridge (*Alectoris rufa*) (Johnston, *et al.*, 1989). Other fungicides of the same class were also shown to

induce the hepatic and duodenal MFO activities of the Japanese quail (Riviere, *et al.*, 1983; Riviere, *et al.*, 1984). In contrast, prochloraz pretreatment in the rat was shown to produce only small increases in hepatic cytochrome P-450 content and activity (Riviere, *et al.*, 1983). Increases in avian hepatic MFO activity have been observed in chick embryos following glucocorticoid treatment (Leakey, *et al.*, 1979). Ethanol and glutethimide were investigated in the chicken embryo and shown to be inducers of hepatic microsomal activity (Sinclair, *et al.*, 1989). A comparative study with aflatoxin B_1 showed that the hepatic cytochrome P-450 content and activity were decreased in the rat and quail but that no change in either parameter occurred in the chicken upon administration of this toxin (Gawai, *et al.*, 1992). Studies which examined the effects of fenbendazole on the hepatic microsomal enzymes found no increase in activity in the rat and chicken while enhanced MFO activity was seen in the quail (Dalvi, 1989; Dalvi, *et al.*, 1991). The pyrethroid pesticides examined did not produce an inductive response in the Japanese quail (Riviere, *et al.*, 1983).

2.3 Cytochrome P-450 Biomarkers in Avian Species

A number of studies have been conducted to evaluate the response of the avian cytochrome P-450 system to chemical exposure and to assess the applicability of utilizing this system as a biomarker in environmental monitoring programs. In general, two approaches have been used for these studies: field investigations and laboratory studies. Field investigations, for the most part, have been based on 3 approaches: (1) determining if correlations exist between contaminant burdens and

MFO activity; (2) monitoring alterations in MFO activity subsequent to a chemical release or the introduction of a migratory species to a contaminated area; and (3) comparing the MFO activities of birds residing in areas of chemical contamination with those of birds obtained from "clean" areas, *i.e.*, controls (Rattner, *et al.*, 1989). Laboratory studies have been directed at characterizing hepatic cytochrome P-450 in avian species with respect to metabolic capacity for environmentally-relevant chemicals, biotransformation mechanisms related to chemical toxicity, changes in the system resulting from exposure to environmental chemicals, and the identification of specific activities for application in biomonitoring programs.

Much of the research that has been conducted on the use of biomarkers in avian species has focused on assessing the response of the MFO system to environmentally persistent constituents such as organochlorines. Early field and laboratory studies provided evidence that these types of pollutants were bioaccumulating in raptor species and that this effect was related to the population decline of such species as the peregrine, sparrowhawk, osprey, and bald eagle (Walker, *et al.*, 1991; Walker, *et al.*, 1987). This work lead researchers to question the ability of these and other birds to metabolize environmental contaminants and subsequently, a number of studies were undertaken to characterize the MFO system of avian species. A variety of species, environmental chemicals and MFO activities were investigated by a number of researchers (Table 2-1). Results of these efforts indicate: (1) there is significant variability in MFO activity within and between avian species; (2) the avian hepatic

MFO system is readily induced by a range of environmental pollutants and a number of avian hepatic MFO activities have potential use as biomarkers of environmental exposure; (3) laboratory data indicated that MFO activity is correlated with tissue residues and other toxic effects in avian species; and (4) the monitoring of MFO activity in developmental stages, such as embryos and nestling, offers a number of advantages when using avian species in biomonitoring programs.

2.3.1 Variability of MFO Activity

High variability in MFO activity between avian species, as well as among individuals within a given species, has been documented by a number of researchers (Boersma *et al.*, 1986; Fossi, *et al.*, 1990; Husain, *et al.*, 1981; Knight and Walker, 1981; Peakall, *et al.*, 1987; Rattner *et al.*, 1989; Walker, *et al.*, 1981). Variations in genetic constitution, physiological factors, substrate specificity, and environmental influences make it difficult to compare cytochrome P-450 levels and activities between avian species (Riviere, *et al.*, 1985). Profound interspecies differences in avian MFO activity have been observed between the predators and other species of birds. This dramatic difference in MFO activity has been suggested to be related to the difference in feeding ecology of the two groups (Walker *et al.*, 1987). Predator species have narrow dietary ranges which are postulated to result in limited xenobiotic exposure and, thus, a reduced need for detoxification by the MFO system. For example, piscivorous birds, such as the razorbill (*Alca torda*), puffin (*Fratercula arctica*), shag

Table 2-1

Avian Species and Cytochrome P-450 Parameters Examined in Ecotoxicological Studies

Species	Potential Exposure, Pretreatment, or Tissue Residue	Parameter Characterized[1]	Reference
Puffin	DDE	ANH, BDM	Bend *et al.*, 1987
Herring Gull, Cormorant, Eider Duck	PCBs	EROD, P-450	Brouwer, 1991
Forster's Tern	PCBs, TCDD, PCDD	AHH	Hoffman, *et al.*, 1987
Puffin	Crude Oil	EROD, AHH, AE	Peakall, *et al.*, 1987
Puffin, Razorbill, Guillemot, Shag, Cormorant, Manx Shearwater	PCBs	HCE, AE	Knight *et al.*, 1981 Walker *et al.*, 1982a
Black-Headed Gull, Herring Gull, Rook	None	HCE, AE	Knight *et al.*, 1981 Walker *et al.*, 1982a
Herring Gull	Organochlorines	AHH	Ellenton, *et al.*, 1985
Herring Gull	Organochlorine Pesticides and PCBs	AHH, APDM, EROD, P-450	Peakall, *et al.*, 1986
Herring Gull	Organochlorine Pesticides and PCBs	APDM, ANH, EROD	Boersma, *et al.*, 1986
Black-crowned Night Heron	Organochlorine Pesticides and PCBs	AHH	Hoffman, *et al.*, 1986

(table continued)

Table 2-1

Avian Species and Cytochrome P-450 Parameters Examined in Ecotoxicological Studies

Species	Potential Exposure, Pretreatment, or Tissue Residue	Parameter Characterized[1]	Reference
Pigeon	Organochlorine Pesticides	AHH	Husain, *et al.*, 1981
Robin	Dioxins	AHH	Martin, *et al.*, 1987
Black-necked Grebe	Organochlorine Pesticides and PCBs	AE, EROD	Fossi, *et al.*, 1986
Black-headed Gull	PCBs	AE, EROD	Fossi, *et al.*, 1986
Pigeon, Crow, Kite, Vulture, Cattle Egret	None	AHH, APDM	Husain, *et al.*, 1981
Mallard Duck	Crude Oil	AHH, P-450	Gorsline, *et al.*, 1981
Barn Owl, Chicken	PCBs	APDM, AE, P-450	Rinkzy and Perry 1983
Chicken	PCBs	AHH, EROD	Rifkind, *et al.*, 1984
Chicken	Crude Oil	AHH, EROD	Lee, *et al.*, 1986
Chicken	PCBs, DDT	ANH, APDM	Cecil, *et al.*, 1978
Chicken	Organophosphate Pesticide	ANH, CADM	Varshneya, *et al.*, 1986
Japanese Quail	Pyrethroids, Dicarbomide Fungicide	AE, ANH, ECOD, EROD, P-450	Riviere, *et al.*, 1983

(table continued)

Table 2-1

Avian Species and Cytochrome P-450 Parameters Examined in Ecotoxicological Studies

Species	Potential Exposure, Pretreatment, or Tissue Residue	Parameter Characterized[1]	Reference
Japanese Quail, Chicken, Pheasant, Partridge	Prochloraz	AE, ANH, EROD, P-450	Riviere, *et al.*, 1985
Pigeon	None	AE, HCE	Ronis and Walker, 1985
Pigeon	Organochlorines	AHH	Kaphalia, *et al.*, 1981
Kestral, Sparrowhawk	None	AE, HCE	Walker, *et al.*, 1987
Herring Gull	Crude Oil	AHH, ANH, APDM, EROD	Lee, *et al.*, 1985
Japanese Quail, Chicken, Mallard Duck	DDT	ANH, APDM	Sifri, *et al.*, 1975

[1]AE, aldrin epoxidase; AHH, aryl hydrocarbon hydrolase; ANH, aniline hydroxylase; APDM, aminopyrine demethylase; BDM, benzphetamine demethylase; CADM, *p*-chloromethyl aniline demethylase; ECOD, ethoxycoumarin dealkylase; EROD, ethoxyresorufin-*O*-deethylase; HCE, 1,2,3,4,9,9-hexachloro-1,4,4a,5,6,7,8,8a-octahydro-exo-7,8-epoxy-1,4-methanonaphthalene hydroxylase; P-450, cytochrome P-450 level

(*Phalacrocorax aristotelis*), cormorant (*Phalacrocorax carbo*), and guillemot (*Uria aalge*), have been shown to have MFO activities that are considerably lower than those of other avian species and in fact, activities have been noted to be comparable to those in fish (Knight and Walker, 1981; Walker, *et al.*, 1987). Omnivorous birds, on the other hand, have wide dietary ranges which are thought to result in a more varied exposure to xenobiotics thereby creating a need for a more active MFO system (Walker, *et al.*, 1987; Knight and Walker, 1981). It has been argued that the hepatic cytochrome P-450 systems of birds with complex diets are more developed and have greater metabolic capacity than birds with specialized diets (Ronis and Walker, 1989). One exception to this finding is the puffin (*Fratercula arctica*). Although the puffin is a fish-eating bird, MFO activity (for organochlorine substrates) in this species has been shown to be greater than other avian species and is reported to be comparable to that seen in the rat (Knight and Walker, 1981).

Potential sources of intraspecies variation in MFO activity include age, sex, and reproductive status. With regard to age, changes in MFO activity have been documented in the herring gull (*Larus argentatus*), chicken, barn owl, kestrel (*Accipiter nisus*), and sparrowhawk (*Falco tinnunculus*) during various stages of development. Ellenton and co-investigators (Ellenton, *et al.*, 1985) found that AHH and EROD activities increased with embryonic development whereas aminopyrine demethylase (APDM) activity decreased. In addition, AHH activity was more inducible at day 25 of incubation than at day 20. No consistent changes were

apparent for other substrates. In nestlings, no consistent changes in MFO activity were noted in this study, but activity was higher than that of the embryos. Peakall and co-workers (Peakall, *et al.*, 1986) found that APDM activity decreased with age in herring gull (*Larus argentatus*) nestlings while no change was noted in EROD or AHH activities or cytochrome P-450 levels. In chickens, MFO activity has been demonstrated to be inducible at day 17 of incubation with constitutive activity peaking at day 1 following hatching (Ellenton, *et al.*, 1985; Rinzky and Perry, 1983). In contrast, MFO activity in the barn owl was found to be lower at day 1 post-hatch than at any other age studied (Rinzky and Perry, 1983). In the sparrowhawk and kestrel, MFO activities were found to be lower in juvenile birds than in adults (Walker, *et al.*, 1987). In another study, Ronis noted that aldrin epoxidase activity increased with age in cockerels (Walker, *et al.*, 1987).

No significant differences in MFO activity between males and females have been observed for the puffin (*Fratercula arctica),* Leach's petrel (*Oceanodroma leucorhoa*), or herring gull (*Larus argentatus*) (Knight and Walker, 1982; Peakall, *et al.*, 1986; Peakall, *et al.*, 1987). Also, no sex differences were demonstrated in the chicken embryo (Hamilton *et al.*, 1983), chicken hatchling (Brunstrom, 1986), or in the adult wild pigeon (Husain *et al.*, 1984). However, an increase in MFO activity has been reported in the female razorbill (*Alca torda*) at the onset of the breeding season and it is speculated that, in light of the known involvement of the MFO system in steroid metabolism, this increase is associated with the elevation of steroid levels that occurs

at the time of egg laying (Knight and Walker, 1982a). Similar findings have been reported in the black-necked grebe (*Podiceps nigricollis*) (Fossi, *et al.*, 1986), wood pigeon (*Columba palumbus*), yellow-legged herring gull (*Larus argentatus cachinnans*) and herring gull (*Larus argentatus smithsonainus*) (Fossi, *et al.*, 1990). Differences between the sexes have been noted in the guillemot (*Uria aalge*) and cormorant (*Phalacrocorax carbo*) in which MFO activity in the female was half that in the male (Walker and Ronis, 1989). Sell and coworkers (1971) noted an inverse relationship between MFO activity and clutch length in the chicken. Rao and McKinley (1969) observed a difference in the rate of oxidation of organophosphorus insecticides in male and female chickens, however, no difference was seen between the sexes for the demethylating system.

It is interesting to note, that while high variability in MFO activity is observed between avian species, little variation is seen in cytochrome P-450 content (Knight and Walker, 1981). In addition, very little individual variation in MFO activity has been observed in the sparrowhawk, a specialized predator species with very low MFO activity (Walker, *et al.*, 1987). The data available to date clearly indicate the importance of characterizing the potential sources of variation in the hepatic MFO activity of target species. It is imperative that these factors be taken into consideration when planning biomonitoring programs based on MFO biomarkers (Peakall, *et al.*, 1987; Rattner *et al.*, 1989).

2.3.2 Induction of MFO Activity

Characterization of the induction response of the cytochrome P-450 system is an essential area of research for the identification of specific enzyme activities to serve as biomarkers of exposure in avian species. The avian hepatic microsomal MFO has been demonstrated to be inducible by a number of environmental contaminants (Pan and Fouts, 1978). Polychlorinated biphenyls (PCBs), common environmental contaminants, have been demonstrated to induce APDM and aldrin epoxidation (AE) activities in the barn owl (Rinsky and Perry, 1983) and EROD activity in the Eider duck (*Somateria mollissima*) (Brouwer, 1991). Other persistent organochlorine compounds that have been reported to induce the hepatic MFO in avian species include TCDD [American robin (*Turdus migratorius*), AHH and EROD activities (Martin, *et al.*, 1987)]; DDE [puffin (*Fratercula arctica*), aniline hydroxylase (ANH) activity (Bend, *et al.*, 1977)]; and *p,p'*-DDT [Mallard duck (*Anas platyrhynchos*), ANH and APDM activities (Sifri, *et al.*, 1975)]. However, chicken (*Gallus domesticus*) MFO was not shown to be induced by PCBs (Rinsky and Perry, 1983) nor by *p,p'*-DDT (Sifri, *et al.*, 1975). In fact, suppression of MFO activity has been noted in the chicken following exposure to *p,p'*-DDT (Sell, *et al.*, 1971; Sifri, *et al.*, 1975; Stephen, *et al.*, 1971). Similar results were found by Varshney and co-workers (1986) following exposure of the chicken to malathion, an organophosphate pesticide. In another Galliformes species, the Japanese quail (*Courtnix japonica*), inhibition of ANH and APDM activities were noted following pretreatment with *p,p'*-DDT (Sifri, *et al.*, 1975). Other researchers have also observed decreases in MFO activity in

Japanese quail following DDT pretreatment (Bunyan, *et al.*, 1972; Cecil, *et al.*, 1978; Gillette and Arscott, 1969; Sell, *et al.*, 1972). Exposure to another important class of environmental compounds, PAHs, has been shown to induce MFO activity in a variety of avian species. Various types of crude oil administered to chickens (Lee, *et al.*, 1986), herring gulls (*Larus argentatus*) (Lee, *et al.*, 1985), mallard ducks (Gorseline, *et al.*, 1981), and puffins (Peakall, *et al.*, 1987) produced significant increases in the MFO activities of these species. Induced activities included AHH and EROD in the chicken; AHH, EROD, ANH, and APDM in the herring gull, AHH in the mallard; and AHH, EROD, and AE in the puffin. Prochloraz, an agricultural fungicide, has been documented as an efficient inducer of the MFO system of four Galliformes species: the Japanese quail, chicken, partridge (*Perdix perdix*), and pheasant (*Phasianus colchicus*) (Riviere, *et al.*, 1985). Specific activities induced in these birds included EROD, ANH, and AE. *N*-(3,5-dichlorophenyl) dicarboximide, another fungicide, has also been shown to induce the MFO system of the Japanese quail (Riviere, *et al.*, 1983). In field investigations based on comparisons of MFO activity in herring gull embryos collected from areas of organochlorine pesticides and TCDD contamination with embryos obtained from nonimpacted control areas, MFO activity in the exposed group demonstrated elevated AHH and EROD activities, depressed ANH activity, and similar APDM activity compared to the control group (Ellenton, *et al.*, 1985; Boersma *et al.*, 1986). In a similar study, Hoffman and co-workers (Hoffman, *et al.*, 1987) found elevated AHH activity in Forster's tern embryos exposed to PCBs, TCDD, and dibenzo-*p*-dioxins.

As discussed above, a number of avian hepatic microsomal MFO activities have been demonstrated to be feasible biomarkers of environmental chemical exposure. In summary, the activities that have been most commonly examined in avian species for the assessment of environmental contamination include EROD, AHH, AE, APDM, and ANH. In field investigations, the most frequently monitored MFO activities include EROD and AHH. Among the species studied, the most consistent change in MFO activity following chemical exposure (PCBs, organochlorines, TCDD, crude oil), under laboratory and field conditions, was the induction of EROD and AHH activities. Laboratory studies indicate that HCE, aminopyrine and aldrin are relatively poor substrates for avian monooxygenases whereas ethoxyresorufin and aniline are relatively good substrates. Aniline, aldrin, aminopyrine and HCE are metabolized by a variety of P-450 isoforms and therefore are rather non-specific substrates in avian species. On the other hand, ethoxyresorufin is a specific substrate with metabolism being primarily attributable to the P-450 isoform (CYP1A1) inducible by 3MC-type inducers. In addition, the metabolic capacity of avian monooxygenases has been demonstrated to be much higher for ethoxyresorufin than with other non-specific substrates (Ronis and Walker, 1989).

2.3.3 Correlation of MFO Activity With Tissue Burden

An important area of ecotoxicological investigation has been the study of the potential relationship between tissue burdens and MFO activity in exposed birds. MFO activity is inversely related to the biological half-life of xenobiotics, and, thus, plays an

important role in determining the degree of bioaccumulation of persistent environmental pollutants and the potential susceptibility of certain avian species to xenobiotics that are detoxified by this system (Ronis and Walker, 1985). Low MFO activity in certain avian species towards such xenobiotics, as in the piscivorous birds, has been correlated with a marked tendency for these compounds to bioaccumulate in exposed birds (Knight and Walker, 1981; Walker *et al.*, 1987). The correlation of MFO activity with specific chemical residue levels is used in field studies to determine if MFO induction is related to environmental chemical exposure (Peakall, *et al.*, 1980). However, to date, such field studies have been met with limited success. A study on herring gull (*Larus argentatus*) embryos obtained from areas of environmental contamination was conducted by Ellenton and co-investigators (Ellenton *et al.*, 1985) to determine if there was a correlation between hepatic MFO activity and tissue residues in the exposed birds. The study demonstrated a significant positive correlation between the induction of AHH activity and TCDD tissue residues; however, MFO activity was not found to be correlated with other organochlorine (PCBs and DDE) residues present in the embryos. In a similar study conducted by Boersma and co-workers (1986), negative correlations were noted between APDM activity and PCB residues, APDM activity and hexachlorobenzene residues, and ANH activity and mirex residues in herring gull embryos. Peakall and co-workers (1986) examined the MFO activity and tissue burdens of nestling Herring gulls. No correlations were apparent between AHH, EROD, and AMPD activities and any of the organochlorine residues detected. However, cytochrome P-450 levels were

significantly correlated with levels of hexachlorobenzene and oxychlordane present in the tissues. Also, APDM and AHH activities were significantly correlated with each other suggesting that these enzymes are potential alternates for biological effects monitors (Peakall, *et al.*, 1986). Other studies conducted on black-crowned night herons (*Nycticorax nycticorax*) (Hoffman *et al.*, 1986), razorbills (*Alca torda*), puffins (*Fratercula arctica*) (Knight and Walker, 1982) and black-headed gulls (*Larus ridibundus*) (Fossi, *et al.*, 1986) obtained from areas of environmental contamination were not able to demonstrate a correlation between MFO activity (AHH; AE, and HCE hydroxylation; and EROD, and AE; respectively) and tissue burdens (organochlorine compounds). In another type of bioaccumulation study, marked variations in tissue residue levels were observed in puffins administered equal dosages of chemical and returned to the wild (Knight and Walker, 1982a). This intraspecies variability in bioaccumulation is thought to be due to the individual variation in MFO activity that has been documented in the puffin.

2.3.4 Correlation of MFO Activity with Biological Effects

Additional research has focused on the toxicological implications of pollutant tissue residues (organochlorine and PAHs) and MFO induction (primarily *CYP*1A) in exposed birds and the establishment of a causal linkage between exposure and adverse effects observed in exposed avian populations (Gorsline, *et al.*, 1981; Lee, *et al.*, 1985; Lee *et al.*, 1986; and Mineau, *et al.*, 1984). Reproductive failure, congenital malformations, impaired growth and decreased survival have been documented in

avian species inhabiting areas of environmental contamination (Hoffman, *et al.*, 1987; Mineau, *et al.*, 1984; Rattner, *et al.*, 1989; and Walker and Ronis, 1989). Furthermore, laboratory studies have demonstrated that induction of the hepatic microsomal MFO system interferes with steroid metabolism in the mallard duck (*Anas platyrhynchos*), quail (*Corturnix coturnix*), ringdove (*Streptopelia risoria*) (Rattner *et al.*, 1989), and white king pigeon (Peakall, *et al.*, 1967). However, at the present, no direct evidence has been obtained from field studies to support the contention that MFO induction resulting from environmental pollution is associated with these biological effects (Rattner *et al.*, 1989).

2.3.5 Use of Avian Developmental Stages in Biomonitoring Programs

Birds of various ages and stages of embryonic development have been utilized for bioeffects monitoring. Adult birds have been used extensively in ecotoxicological and MFO research. As previously discussed, significant variation in MFO activity exists among individual birds within a given species and a number of sources of variation have been identified in certain avian species. Consequently, this variability has complicated the elucidation of exposure and induction relationships in adult birds (Rattner, *et al*,. 1989). The use of MFO induction as a biomarker of chemical exposure in adult birds requires further characterization of the MFO system and the those factors that influence it. Avian embryos are frequently chosen for use in field and laboratory studies for several reasons: egg collection is relatively simple and produces minimal disturbance within the population (Ellenton, *et al.*, 1985; Mineau

et al., 1984); individual variation in MFO activity potentially associated with differences in age, sex and reproductive status are reduced or eliminated; the hepatic MFO system of the embryo is readily induced (Ellenton, *et al.*, 1985); there is less variation in the lipid content, and thus in tissue burdens, of eggs than in other tissues; and the tissue burdens of eggs are comparable to those of the laying adult (Mineau *et al.*, 1984). However, it should be noted that the removal of an embryo from its natural environment or the incubation of an embryo *in vitro* can profoundly affect the activities of the hepatic MFO enzymes (Hamilton, *et al.*, 1983). Nestlings are also frequently used in biomonitoring research. Nestlings are more easily collected than adults and more readily adapt to captivity for experimental purposes. Also, the collection of juveniles is less destructive to the population than the removal of adults since the mortality of nestlings and embryos is much greater than that of adults (Peakall, *et al.*, 1986). Based on the data available to date, the monitoring of MFO activity in embryos and hatchlings offers promise as a biomarker of chemical exposure in avian species (Rattner, *et al.*, 1989).

2.4 Representative Avian Species

Birds are classified into orders based on structural and anatomical likenesses. Within each order, birds are further classified into families based on the distinguishing characteristics of the species that have allowed for adaptation to a particular environment and way of life (Terres, 1980). Two orders of birds are represented in this study, the Galliformes and Anseriformes. The Galliformes include the chicken-

or fowl-like terrestrial birds which are considered as game birds. Galliformes primarily feed on the ground obtaining seeds, grain, fruit, buds, nuts and vegetable matter as well as insects, spiders, snails, worms and other invertebrates. The Galliformes order is represented in this study by the chicken (*Gallus gallus* or *Gallus domesticus*), turkey (*Melegris gallopavo*) and Japanese quail (*Coturnix coturnix*). The domestic chicken belongs to the Phasianidae (Pheasant) family and is a descendant of the red jungle fowl which originated from Southeast Asia and India. The White Leghorn, which was utilized in this study, is classified as a Mediterrean breed (Phillips, *et al.*, 1985). The Japanese quail is also a member of the Phasianidae family and is classified as an Old World species. This migratory bird is a native of Europe, Central Asia, Africa, and Japan (Terres, 1980; Reilly, 1968). The domestic turkey belongs to the Meleagrididae family and is a descendant of a cross between the Mexican wild turkey and the native turkeys of North America (Phillips, et al., 1985 and Terres, 1980). The turkey is a gregarious, nonmigratory species found in wooded areas (Reilly, 1968).

The Anseriformes order includes the waterfowl (or wildfowl) which are classified as migratory game birds. Waterfowl are an ancient group of birds with their oldest fossil remains dating back 80 million years. Some orthinologists believe that the waterfowl evolved from an ancestor related to the gallinaceous birds (Terres, 1980). These birds are found on every continent but Antarctica. The Anseriformes represented in this study include the duck (*Anser platyrhynchos*), represented by the Khaki Campbell and

Pekin breeds, Muscovy duck (*Cairina moschata*), and goose (*Anser anser*). Each of these species is member of the Anatidae family. The Khaki Campbell and Pekin ducks were derived from the wild mallard duck in England and China, respectively (Kear, 1985 and Terres, 1980). These birds are classified as dabbling or surface-feeding ducks and are found in marshy habitats. They are gregarious feeders and consume primarily aquatic plants (Terres, 1980). The Muscovy duck is a native of Mexico and Central and South America (Terres, 1980) and is related to the Mandarin and Carolina (or North American Wood Duck) ducks (Kear, 1985). This species is classified as a perching duck due to their ability to perch on branches and similar structures. The Muscovy duck is nonmigratory and is found as a permanent resident of coastal bottomland timbers along waterways and marsh lands. These birds feed on small fish, aquatic insects, mollusks, aquatic plants, grain, seeds, reptiles, worms and snails (Terres, 1980). The Muscovy duck is not considered by many breeders to be a true duck due to a number of unique characteristics exhibited by this species. Unlike other ducks, the Muscovy cannot spend extended amounts of time on the water because their feathers are not as water repellant as those of true ducks and they may become soaked, causing the ducks to drown. They are also less tolerant of wet and cold weather. When crossed with other species of ducks, the offspring are typically sterile. Also, as mentioned above, they are perching ducks (Holderread, 1978). The domestic goose is a descendant of the wild European Greylag goose (Terres, 1980). These birds are gregarious and nest in large colonies. Their diets exist primarily of plant materials and they frequently graze on land (Terres, 1980; Reilly, 1968).

The most important species from an economic point of view are the domestic chicken, turkey and duck. The chicken and turkey are produced commercially for both egg and meat production. The Khaki Campbell duck is utilized for egg production while the Pekin and Muscovy are utilized for meat production. The quail is raised commercially for both egg and meat production and the goose is raised primarily for meat production (Phillips, et al. 1985).

3.0 EXPERIMENTAL GOALS AND DESIGN

This study was conducted to characterize the oxidative biotransformation capacity of the hepatic microsomal system of selected avian species representing the Galliformes and Anseriformes orders. The hepatic cytochrome P-450 systems of seven avian models and a mammalian model were investigated. The Galliformes order was represented by the chicken, turkey and Japanese quail. The Anseriformes order was represented by the goose, Pekin duck, Khaki Campbell duck, and Muscovy duck. The male rat was utilized for the mammalian model. Two groups of animals for each species were evaluated and these consisted of a control group, which received no treatment, and a treatment group which was administered β-naphthoflavone (50 mg/kg) intraperitoneally once a day for 3 days (Figure 3-1) prior to microsomal isolation on the fourth day. Each group consisted of six animals and investigations were conducted on hepatic microsomes prepared from individual animals.

The goal of this research was to initiate a database on the hepatic cytochrome P-450-dependent mixed function monooxygenases of domestic avian species for the purpose of addressing two objectives. The first objective of this study was to obtain fundamental information on the cytochrome P-450-dependent metabolic capacity of certain economically major and minor food-producing avian species for a given class of xenobiotics and to characterize the differences in metabolism of a model compound between the species. The goal of this objective was to provide biotransformation information relating to the hepatic cytochrome P-450 system of major and minor

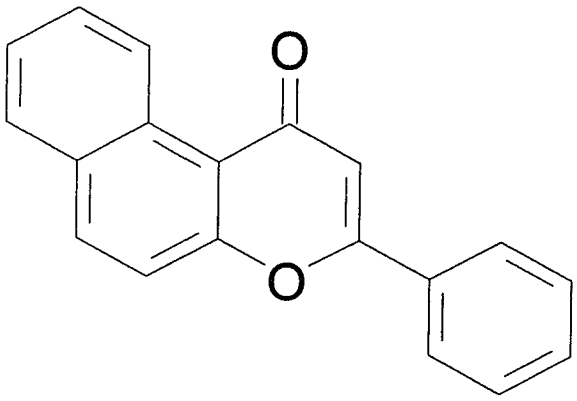

Figure 3-1 Structure of beta-naphthoflavone.

6species to support the interpretation of data submitted to the FDA for the purpose of obtaining approval for minor use avian drugs. For this study, the chicken and turkey represented economically major species while the Japanese quail, Pekin duck, Khaki Campbell duck, Muscovy duck and goose represented minor species. The second objective of this study was to characterize selective constitutive and inducible isozymes of the avian hepatic cytochrome P-450 system, determine the inducibility of the system utilizing a classical inducer of environmental relevance and investigate the metabolism of an environmentally relevant compound via the quantitation of individual metabolites. The goal of this objective was to evaluate the potential use of the representative avian species as sentinel organisms utilizing induction of the hepatic cytochrome P-450 system as a biomarker of environmental chemical exposure.

To achieve these objectives a series of investigations were undertaken:

(1) The components of the avian cytochrome P-450 system were quantitated including microsomal protein yield, cytochrome P-450 content, NADPH- and NADH-cytochrome *c* reductase activities.

(2) Ethoxyresorufin-*O*-deethylase activity (Figure 3-2) was determined to characterize the response of the avian hepatic cytochrome P-450 system to β-naphthoflavone treatment. Presently, five isozymes of cytochrome P-450 have been isolated from hepatic microsomes of rats treated with

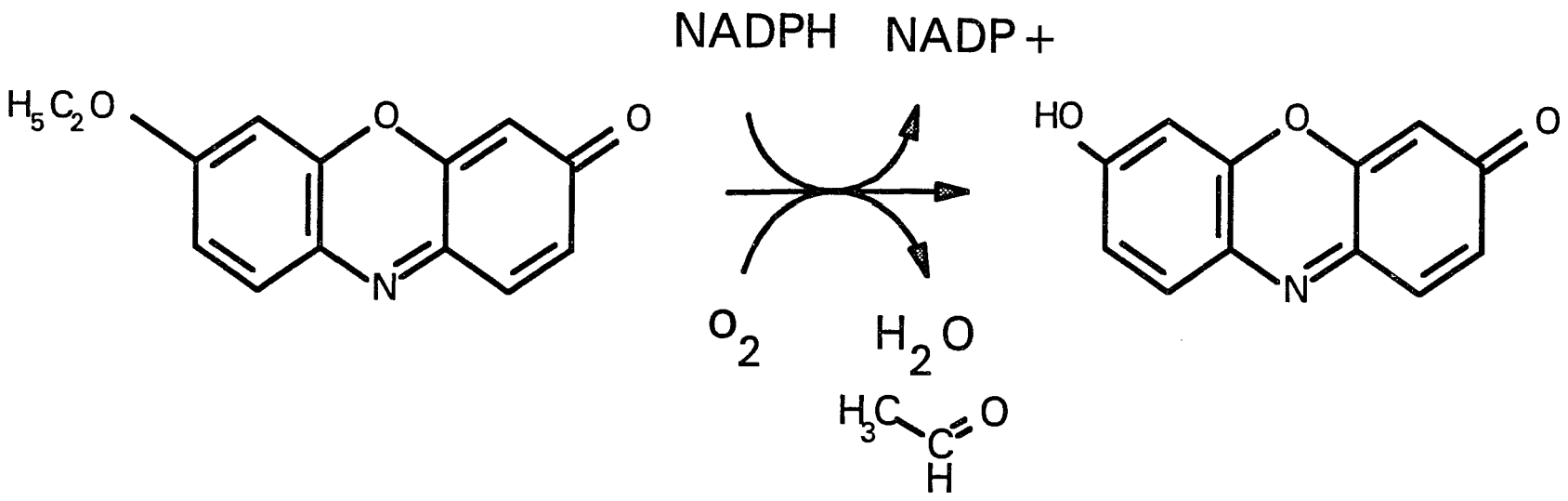

Figure 3-2 O-deethylation of ethoxyresorufin.

β-naphthoflavone (and other 3MC-type inducers). Of these, two major forms, CYP1A1 and CYP1A2 have been identified (Ioannides and Parke, 1987; Lau and Strobel, 1982; Sesardic *et al.*, 1990; Thomas, *et al.*, 1983). Ethoxyresorufin (an alkoxyphenoxazone derivative) has been demonstrated to be an isozyme-selective substrate for the CYP1A1 isozyme and an increase in the *O*-deethylation of ethoxyresorufin has been shown to be associated with induction of this isozyme (Burke, *et al.*, 1985; Burke and Mayer, 1975; Guengerich, *et al.*, 1982). The CYP1A1 and CYP1A2 isozymes have been shown to be inducible by certain classes of environmental pollutants in laboratory animals and man. In addition, they have been demonstrated to increase the toxicity of a number of compounds via the formation of highly reactive intermediates (Sesardic, *et al.*, 1990).

(3) The hepatic microsomal oxidative biotransformation of benzo[*a*]pyrene was examined. Four metabolites, 3-hydroxybenzo[*a*]pyrene, 9-hydroxy-benzo[*a*]pyrene, benzo[*a*]pyrene-7,8-dihydrodiol, and benzo[*a*]pyrene-9,10-dihydrodiol, were identified and quantitated by HPLC analysis. These metabolites were selected based the pathways of hepatic oxidative metabolism identified in control and in βNF-treated chicken embryos and rats (Figure 3-3). In primary cultures of chick embryo hepatocytes, Topp and van Bladeren (1986) observed the formation of tetrahydrotetrols, 3-hydroxybenzo[*a*]pyrene, 9-hydroxybenzo[*a*]pyrene, benzo[*a*]pyrene 7,8-

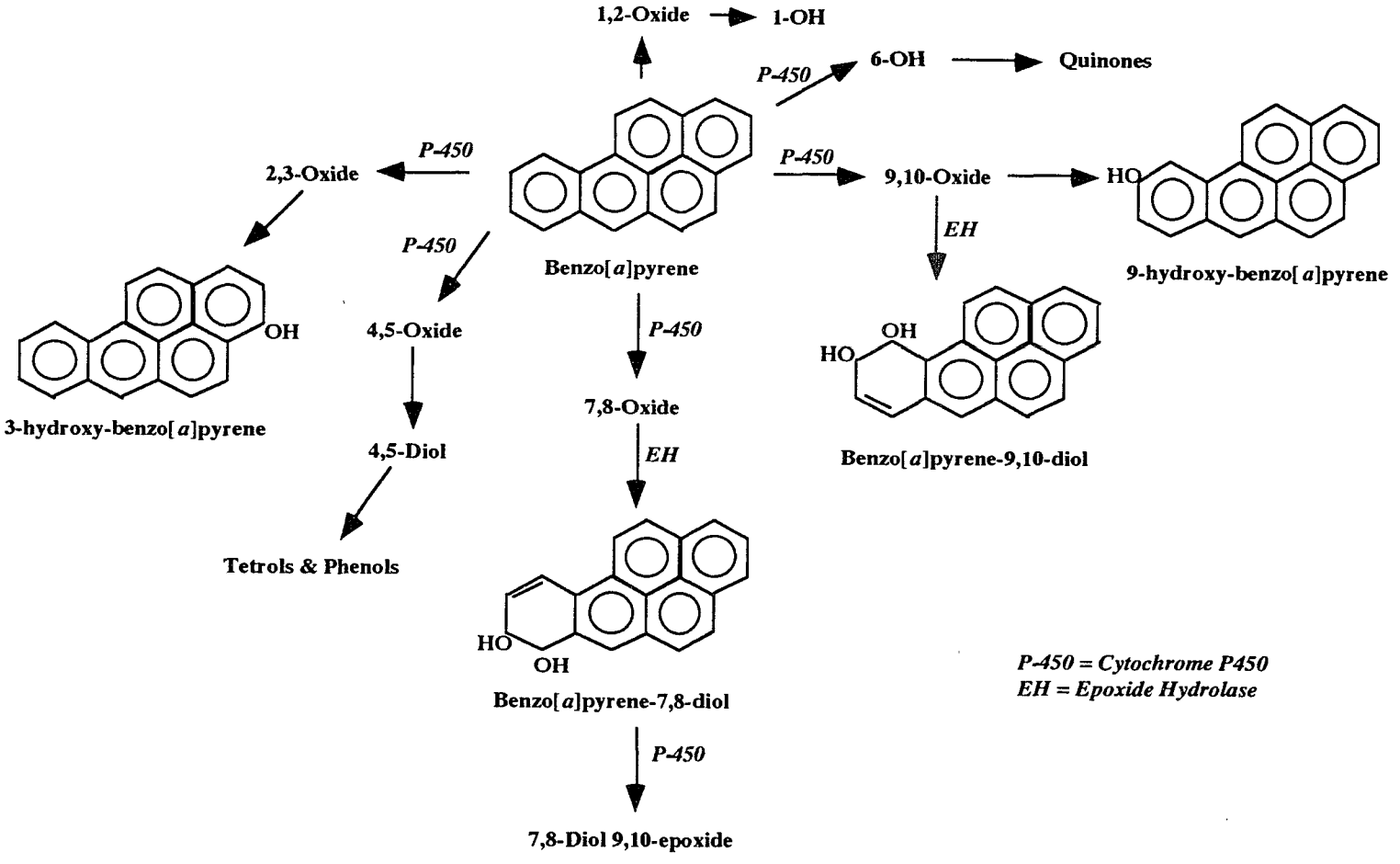

Figure 3-3 Pathways of benzo[*a*]pyrene metabolism (Grover, 1986).

dihydrodiol, and benzo[*a*]pyrene 9,10-dihydrodiol by control and βNF-treated cells. Control and induced rat hepatic microsomes have been shown to metabolize B[*a*]P to a variety of phenols, dihydrodiols, and quinones. The major metabolites formed in the rat include 3- and 9-hydroxybenzo[*a*]pyrene, benzo[*a*]pyrene 4,5-, 7,8-, and 9,10-dihydrodiols and benzo[*a*]pyrene 1,6-, 3,6-, and 6,12-diones (Cavalieri, *et al.*, 1988; Grover, 1986; Jongeneelen, *et al.*, 1985; Levin, *et al.*, 1982; Winston, *et al.*, 1991; Wong, *et al.*, 1986). Benzo[*a*]pyrene was selected as a substrate for this metabolic study for two reasons. First, the oxidative metabolic pathways of this compound have been well described in a number of species. Secondly, benzo[*a*]pyrene is a representative substrate of an environmentally important class of compounds, the polycyclic aromatic hydrocarbons. The βNF-inducible isozymes CYP1A1 and CYP1A2 have both been demonstrated to be involved in the metabolism of benzo[a]pyrene in the rat but CYP1A1 has been shown to be approximately 50 times more active than CYP1A2.

These investigations of the cytchrome P-450 system were conducted on hepatic microsomes of both control and βNF-treated birds to determine the effects of induction on cytochrome P-450 content, catalytic activity and pathways of oxidative metabolism. Comparisons were sought between the avian species, between avian orders, and between avian species and the rat. Ethoxyresorufin-*O*-deethylase and

benzo[*a*]pyrene hydroxylase activities were evaluated based on specific activity (pmoles of product produced/min/mg of microsomal protein) and on molar activity (pmoles of product produced/min/nmole of cytochrome P-450 also known as turnover number).

4.0 MATERIALS AND METHODS

4.1 Animals

The avian species used in this study included the White Leghorn chicken (*Gallus gallus* or *Gallus domesticus*), Japanese quail (*Corturnix coturnix*), turkey (*Melegris gallopavo*), goose (*Anser anser*), duck (*Anser platyrhynchos*) represented by the Pekin and Khaki Campbell breeds, and Muscovy duck (*Cairina moschata*). Twelve adult female birds of each species and breed were obtained from local vendors, with the exception of 2 quail, which were obtained from the Department of Veterinary Science, Louisiana State University, Baton Rouge, Louisiana. The birds were group-housed by species in enclosed outdoor stalls containing dried hardwood shavings purchased from Purvis Forest Products of Carmen, Mississippi. The quail were kept in groups in small metal cages with grid mesh bottoms and were housed within a stall. All birds received tap water and a 50:50 ration of cracked corn and Purina Layena mash (obtained from Orcola, Louisiana) *ad libitum*. The birds were acclimated for 2 weeks prior to being utilized in the study. Twelve male Sprague-Dawley rats, weighing approximately 200 g, were obtained from Hilltop Lab Animals, Inc. (Scottsdale, Pennsylvania). The rats were group caged in a controlled environment defined by a temperature of 21-24° C, humidity of 40-60% and a light/dark cycle of 12 hours. The rats were given water and Purina rat chow free choice.

4.2 Chemicals

Ethoxyresorufin (resorufin ethyl ether) (98%) was obtained from Molecular Probes, Inc. (Eugene, Oregon). Resorufin (75% dye content) was purchased from Aldrich Chemical Company, Inc. (Milwaukee, Wisconsin). β-Naphthoflavone (90-95%), cytochrome c type III from horse heart (95-100%), NADPH (β-nicotinamide adenine dinucleotide phosphate, tetrasodium salt, type III, reduced form) (98%), NADH (β-nicotinamide adenine dinucleotide, reduced form) (98%), glucose-6-phosphate dehydrogenase (crystalline suspension in 3.2M $(NH_4)_2SO_4$, pH7, activity 300-400 units/mg protein), glucose-6-phosphate (crystalline, monosodium salt), nicotinamide adenine dinucleotide phosphate (sodium salt) (98-100%), and benzo[*a*]pyrene (98%) were purchased from Sigma Chemical Company (St. Louis, Missouri). 3-Hydroxybenzo[*a*]pyrene (> 98% purity by HPLC), 9-hydroxybenzo[*a*]pyrene (> 98% purity by HPLC), benzo[*a*]pyrene-trans-7,8-dihydrodiol(-) (> 99% purity by HPLC), and benzo[*a*]pyrene-trans-9,10-dihydrodiol (> 99% purity by HPLC) were obtained from the NCI Chemical Carcinogen Repository, Midwest Research Institute (Kansas City, Missouri). Water used for the HPLC solvent system was triple-distilled and passed through a Modular Polisher I water purifying system (Continental Water Systems Corp., San Antonio, Texas). All other chemicals and biochemical agents used in this study were obtained from commercial suppliers and were of a quality that met or exceeded analytical grade standards.

4.3 Treatment

A suspension of β-naphthoflavone (5,6-benzoflavone) was prepared in peanut oil at a concentration of 50 mg/ml. The suspension was stored a 4° C and brought to room temperature prior to use. The birds and rats were randomly divided into control and treatment groups consisting of six animals each. The treatment groups were administered 50 mg/kg β-naphthoflavone intraperitoneally once a day for three consecutive days and the animals were sacrificed on the fourth day. The control groups received no treatment.

4.4 Microsomal Preparation

The animals were sacrificed by decapitation and microsomes were prepared from livers of individual animals. All steps involved in the microsomal preparation were carried out at 4° C. The livers were removed, weighed and placed in 1.15% M KCl. The livers were minced with scissors, rinsed with 1.15% M KCl and homogenized in STE buffer (0.25M sucrose, 10 mM Trizma HCl and 1 mM EDTA; pH 7.4) at a 1:4 tissue weight/ buffer volume ratio using a Potter-type Teflon-glass motor driven pestle homogenizer. Microsomal pellets were prepared from the homogenates by differential centrifugation (Lake, 1987). The homogenates were centrifuged (Beckman J-21B) for three 10 minute intervals at 600 x *g*, 8000 x *g* and 14,500 x *g*. The post-mitochondrial supernatant fractions were then centrifuged (Sorvall OTD-65 Ultracentrifuge Oil Turbine Drive, Dupont Instruments) at 105,000 x *g* for 60 minutes to obtain the microsomal fractions. The post-105,000 x *g* microsomal pellets were

resuspended in 0.125 M KCl and recentrifuged at 105,000 x *g* for 60 minutes. The final microsomal pellets were resuspended with a glass hand-held homogenizer in a solution of 0.125 M KCl in 20 percent v/v glycerol and stored at -80° C in 1 ml aliquots. Fresh aliquots of microsomes were brought to 4° C just prior to use for each investigation conducted (except for the determination of cytochrome P-450 content which was conducted prior to microsomal storage).

4.5 Protein Determination

The microsomal protein concentrations were determined by the method of Lowry *et al.* (1951). A standard curve was constructed for each set of samples utilizing a series of concentrations of bovine serum albumin (BSA). The microsomal suspensions were diluted 1:100 with triple-distilled water. Four buffer solutions consisting of the following constituents were prepared: Buffer A, Na_2CO_3 (20 mg/l) and NaOH (4 mg/l); Buffer B_1, 2% sodium potassium tartrate; Buffer B_2, 1% $CuSO_4$-$7H_2O$; and Buffer C, 1 part each of Buffers B_1 and B_2 and 100 parts Buffer A. To 0.1 ml of the diluted microsomal suspensions, 2 ml of Buffer C were added and the sample was vortexed. Following a 10 minute incubation period at room temperature, 0.2 ml of Folin reagent was added and the sample was again vortexed. After a 30-minute incubation period at room temperature, the absorbance of each sample at 750 nm was measured with a Shimadzu UV-160 spectrophotometer, and the protein concentration was automatically determined from the standard curve generated by the spectrophotometer. A representative standard curve for the analysis of microsomal

protein concentration using BSA is presented in Figure A-1 in the Appendix. All samples and standard curve BSA solutions were prepared and measured in duplicate. The yield of microsomal protein/g liver was determined by dividing the microsomal fraction of protein (mg/ml) by the microsomal fraction tissue concentration (g of fresh liver/ml) (Lake, 1987).

4.6 Cytochrome P-450 Content

The cytochrome P-450 content of the hepatic microsomes was determined by the method of Omura and Sato (1964a). The microsomal suspensions were diluted 1:20 in 0.1M potassium phosphate buffer (pH 7.5). A few crystals (1-2 mg) of sodium dithionate were added and the solution was gently mixed. A reference and a sample cuvette containing 1 ml of the reduced microsomal solution were placed in a split-beam spectrophotometer (Shimadzu UV-160). A baseline of equal absorbance between the cuvettes was recorded from 350 to 490 nm. The sample cuvette was then saturated with carbon monoxide by gently bubbling carbon monoxide (1 bubble/sec $\approx$ 40 sec) through the solution. A difference spectrum of the reduced cytochrome P-450 bound to carbon monoxide versus the non-bound reduced cytochrome P-450 was then recorded from 350 nm to 490 nm. The difference in absorbance observed at 350 nm relative to 490 nm ($\Delta A_{450\text{-}490}$) and an extinction coefficient of 91 $mM^{-1}cm^{-1}$ (Omura and Sato, 1964a) were used to quantitate the total content of cytochrome P-450 (nmoles of cytochrome P-450/ml of microsomes). This value was divided by the protein concentration of the microsomal suspension (mg/ml) to obtain the specific

content of cytochrome P-450 in units of nmoles of cytochrome P-450/mg of microsomal protein.

4.7 NADH- and NADPH-Dependent Cytochrome *c* Reductase

The activities of the microsomal flavoprotein reductases were determined using cytochrome *c* as an artificial electron acceptor due to the relative difficulty involved in measuring the actual NADH- and NADPH-cytochrome P-450 reductase activities. This assay is based on the reduction of ferric cytochrome c to ferrous cytochrome c which has a characteristic maximum absorption at λ = 550 nm (Lake, 1967). The method of Phillips and Langdon (1962), as modified by Yasukochi and Masters (1976), was used to determine NADH- and NADPH-dependent cytochrome *c* reductase activities. A solution of cytochrome *c* was prepared by combining 31 mg cytochrome *c* type III from horse heart, 33 ml triple-distilled water and 17 ml of 1 M phosphate buffer. For measurement of the NADH-cytochrome *c* reductase activity, the microsomal suspensions were diluted (1:5 for the avian species and 1:10 for the rat) in 1 M phosphate buffer (pH 7.7). For the determination of NADPH-cytochrome *c* reductase activity, the microsomal suspensions were used at full concentration for the avian species and diluted 1:10 in 1 M phosphate buffer (pH 7.7) for the rat. Aliquots (2.5 ml) of cytochrome *c* solution were placed into reference and sample cuvettes. To the cytochrome *c* solution, microsomes (0.005 ml for the birds and 0.01 ml for the rat) were added, the solutions were gently mixed and the cuvettes were place in a dual-beam Shimadzu UV-160 spectrophotometer. The spectrophotometer

was set in the kinetic mode with a lag time of 5 sec, rate time of 75 sec, interval time of 15 sec, and λ = 550 nm. The spectrophotometer was balanced between the two cuvettes. The reaction was initiated by adding 10 μl of freshly prepared NADPH (10 mM) or NADH (10mM) to the sample cuvette and gently mixing the solution. The cuvette was immediately returned to the sample chamber and the change in absorbance over the course of the reaction time was recorded. The ΔA_{550} in conjunction with an extinction coefficient of 21.1 $mM^{-1}cm^{-1}$ (Lake, 1967; VanGelder and Slater, 1962) was used to quantitate NADH- and NADPH-cytochrome *c* reductase activities (nmole of cytochrome *c* reduced/min/ml microsomes). This value was divided by the protein concentration of the microsomes (mg/ml) to obtain the activities in nmole of cytochrome *c* reduced/min/mg of microsomal protein.

4.8 Ethoxyresorufin-*O*-Deethylase

Ethoxyresorufin-*O*-deethylase activity was determined by the method of Lake (1987). Trial assays at varying protein levels were conducted to establish reaction linearity with regard to protein level for the rat and the avian microsomes of the control and βNF treatment groups (Figures A-2 through A-5). The results of the trial assays indicated that the appropriate microsomal protein contents for the reaction mixture were 0.4 mg for the avian species and 0.15 mg for the rat. The reaction mixture (1.8 ml), which consisted of 50 mM tris hydroxy methyl aminomethane (Tris) HCl buffer (pH 8.4), 5 mM $MgSO_4$, 3 units of glucose-6-phosphate dehydrogenase, 5 mM glucose-6-phosphate, 0.5 mM $NADP^+$ and hepatic microsomes, was prepared and

maintained at 4° C until used. The reaction tubes were preincubated at 37° C for 5 minutes and 0.5 μM 7-ethoxyresorufin (0.2 ml 0.5 mM) was added to the mixture to initiate the reaction. The reaction was carried out at 37° C for 10 min in a shaking water bath. The reaction mixture was placed on ice and the reaction was stopped by the addition of 1 ml of zinc sulfate (5% w/v) followed by 1 ml of a saturated barium hydroxide solution. The reaction mixture was centrifuged (Beckman J-21B) for 10 min at 2000 x *g* at 4° C. To 1.5 ml of the supernatant, 3 ml of 0.5 M glycerine-NaOH buffer (pH 8.5) were added and the mixture was vortexed. Resorufin formation was measured on a spectrofluorometer (Farrand Optical Col, Valhalla, New York) with the excitation and emission wavelengths set at 535 nm and 582 nm, respectively, and quantitated using resorufin as a standard. A standard curve was constructed for each set of samples utilizing a series of concentrations of resorufin. A typical resorufin standard curve for the determination of ethoxyresorufin *O*-deethylase activity is presented in Figure A-6 in the Appendix. All samples were run in duplicate.

4.9 Benzo[*a*]pyrene Hydroxylase

Aryl hydrocarbon hydroxylase activity was determined as described by Guengerich (1982). All samples were run in duplicate. Linear enzymatic kinetics based on the formation of 3-OH B[*a*]P, 9-OH B[*a*]P, 7,8-diol B[*a*]P, and 9,10-diol B[*a*]P were determined with regard to protein concentration and incubation time for the avian and rat microsomes (Figures A-7 through A-12 in the Appendix). The reaction mixture

(0.9 ml) consisted of 50 mM potassium phosphate buffer (pH 7.4), 5 mM $MgCl_2$, 1 mM $NADP^+$, 80 μM benzo[*a*]pyrene, and 0.5 mg microsomal protein. Following a 3 min preincubation period at 37° C, the reaction was initiated by the addition of 3 units of glucose-6-phosphate dehydrogenase and 10 mM glucose-6-phosphate (0.1 ml). The reaction mixture was incubated for 20 min at 37° C in a water bath. The mixture was then placed on ice and the reaction was terminated by the addition of 1 ml of cold acetone followed by vortexing. The mixture was extracted twice with ethyl acetate. The extraction procedure included the addition of 1.5 ml of ethyl acetate to the reaction mixture followed by vortexing and then centrifugation at high speed for 5 min. The top layer was removed and retained for analysis. This procedure was then repeated. The extracts were evaporated to dryness with nitrogen and reconstituted in 250 μl of acetonitrile.

Metabolite separation was achieved by high pressure liquid chromatography (HPLC) (Hewlett Packard HP 1090 HPLC system - HP 79994A Chemstation) utilizing a reversed-phase octadecylsilyl derivatized silica (ODS) column (Vydac 201 TP, 5μ, 4.6 mm I.D. x 15 cm) (The Sep/a/ra/tions Group, Hesperia, California) with a replaceable guard column. Column temperature was maintained at 40° C. Trial runs were conducted with standards and microsomal extracts to refine the HPLC parameters for optimization of metabolite separation and detection. The solvent system utilized for the analyses was isocratic and consisted of water and acetonitrile (65:35 v/v) at a flow

rate of 2.0 ml/min. An injection volume of 10 μl was used via an automated injection system.

Detection of B[*a*]P, 3-OH B[*a*]P, 9-OH B[*a*]P, B[*a*]P-trans-7,8-dihydrodiol (-), and B[*a*]P-trans-9,10-dihydrodiol was achieved through the use of a programmable fluorescence detector and a photodiode array detector operating in series. The fluorescence detector was set at an excitation wavelength of 260 nm and an emission wavelength of 420 nm. The photodiode array detector was set at a wavelength of 254 nm with a bandwidth of 20 nm, a reference spectrum of 550 nm with a bandwidth of 100 nm, and a spectrum range of 210-500 nm. Wavelength selection was based on data provided in the NCI Analytical Datasheets.

Benzo[*a*]pyrene and the B[*a*]P metabolites were identified by retention time through the UV diode array detector and the fluorescence detector, respectively. Quantitation of the metabolites was achieved through the use of external standard calculations. Standards were prepared from stock solutions of B[*a*]P and each of the metabolites. The concentration ranges of the standard solutions were based on the anticipated levels of metabolite production as determined by preliminary assay runs for protein and reaction time determinations. Peak areas were used to establish standard curves for each analyte by plotting the integration areas of the generated peaks. Standard curves were established for each compound being analyzed by utilizing a series of four concentrations which were 1.95, 3.90, 5.85 and 7.80 ng/μl for B[*a*]P 9,10-diol; 0.25,

0.50, 0.75 and 1.00 ng/μl for B[*a*]P 7,8-diol; 0.73, 1.46, 2.19 and 2.92 ng/μl for 9-OH B[*a*]P; 1.99, 3.99, 5.98 and 7.93 ng/μl for 3-OH B[*a*]P; and 20.0, 40.0, 60.0 and 80.0 ng/μl for B[*a*]P. A representative standard curve for each analyte and the respective correlation coefficients are presented in Figures A-13 through A-17 in the Appendix.

A method blank was prepared for each analyte to determine if there were any components in the reaction mixture which might interfere with the identification and quantitation of the compounds of interest. Each method blank consisted of 50 mM potassium phosphate buffer (pH 7.4), 5 mM $MgCl_2$, 1 mM $NADP^+$, 80 μM benzo[*a*]pyrene, glucose-6-phosphate dehydrogenase, 10 mM glucose-6-phosphate (1.0 ml total), acetone (1 ml), and 21.46 μg of B[a]P or one of the metabolites. The quantity of analyte selected for the method blank was equivalent to the amount of B[*a*]P used in the aryl hydrocarbon hydroxylase assay. The blank mixture was extracted twice with 1.5 ml of ethyl acetate as described for the B[*a*]P hydroxylase assay. The extract was evaporated to dryness with nitrogen, reconstituted in 250 μl of acetonitrile and analyzed by HPLC. The method blank chromatograms for each analyte are presented in Figures A-18 through A-22 in the Appendix.

A microsomal blank was prepared for each species to determine if there were any microsomal components which may interfere with the identification and quantitation of the compounds of interest. Each microsomal blank consisted of 50 mM potassium

phosphate buffer (pH 7.4), 5 mM $MgCl_2$, 1 mM $NADP^+$, 0.5 mg of microsomal protein, glucose-6-phosphate dehydrogenase, 10 mM glucose-6-phosphate (1.0 ml total), and acetone (1 ml). The blank mixture was extracted twice with 1.5 ml of ethyl acetate as described for the B[*a*]P hydroxylase assay. The extract was evaporated to dryness with nitrogen, reconstituted in 250 μl of acetonitrile and analyzed by HPLC. The chromatograms of microsomal blanks for each species are presented in Figures A-23 through A-30 in the Appendix. These chromatograms and the method blank chromatograms demonstrated that the assay process was relatively free from interfering substances and that the analytical method employed had the ability to adequately resolve and detect the metabolites of interest from the assay/microsomal components.

To determine recovery, a known quantity of each analyte was added to the reaction mixture which consisted of 50 mM potassium phosphate buffer (pH 7.4), 5 mM $MgCl_2$, 1 mM $NADP^+$, glucose-6-phosphate dehydrogenase, 10 mM glucose-6 phosphate and acetone. The mixture was extracted twice with 1.5 ml of ethyl acetate as described for the B[*a*]P hydroxylase assay. The extract was evaporated to dryness with nitrogen, reconstituted in 250 μl of acetonitrile and analyzed by HPLC. The recovery response was then compared to the external standard response to determine the percent recovery of the analytes. The recovery for B[*a*]P and its metabolites was determined to be greater than 95 percent. Percent recovery for B[*a*]P was also determined during subsequent analyses to monitor extraction efficiency.

All analyses were conducted in duplicate. A calibration curve was constructed for each set of samples analyzed. In addition, a method blank was run with each set of samples. Standards were analyzed after every six samples to serve as a check on the resolving capability of the column throughout the run. Representative chromatograms of B[*a*]P and microsomal metabolite formation for each species are presented in Figures A-31 through A-38 in the Appendix.

4.10 Statistical Analysis

Significant differences between control and βNF-treated groups for each species and for each order were determined utilizing the Student's *t* test (SAS,Institute, Inc., 1988). Avian orders were also compared within each treatment group with a Student's *t* test. The *t* test procedure is based on a single comparison of two group means. Three levels of significance, $p < 0.05$, $p < 0.01$, and $p < 0.001$, were used for the *t* test.

Analysis of variance was used to determine the significance of treatment group, species and the interaction of treatment group by species. To test the significance of differences between species within each treatment group, two multiple comparison techniques were employed, Scheffe's test and Tukey's test (SAS,Institute, Inc., 1988). These methods are multiple comparison tests for pairs of group means which arise in an analysis of variance. Scheffe's test is more conservative, *i.e.*, it is more likely not to find a significant difference between groups, than Tukey's test. Three levels of

significance were examined in the statistical analysis of multiple comparisons, $p < 0.05$, $p < 0.01$, and $p < 0.001$. These statistical analyses were conducted utilizing the SAS statistical package version 6.03 (SAS,Institute, Inc., 1988).

5.0 RESULTS

The components of the hepatic microsomal cytochrome P-450 system, including microsomal protein, specific content of cytochrome P-450, NADPH-dependent cytochrome *c* reductase activity and NADH-dependent cytochrome *c* reductase activity, were quantitated for the control and β-naphthoflavone treatment groups of each species. The effects of β-naphthoflavone (βNF) treatment on ethoxyresorufin-*O*-deethylase and benzo[*a*]pyrene hydroxylase activities were evaluated on a basis of specific activity (quantity of product formed per milligram of microsomal protein) and molar activity (quantity of product formed per nmole of cytochrome P-450, also referred to as turnover number) for the representative avian species and the rat. Statistical comparisons were conducted to determine significant differences between:

- Control and βNF-treatment groups of each species,
- Control groups of the avian species,
- βNF-treatment groups of the avian species,
- Control and βNF-treatment groups of each avian order (Galliformes and Anseriformes),
- Control groups of each avian order,
- βNF-treatment groups of each avian order, and
- Avian and mammalian species (rat).

Statistical differences for each of these comparisons are presented for three levels of significance: $p < 0.05$, $p < 0.01$, and $p < 0.001$. The acronyms utilized to designate the species and treatment group are presented in Table 5-1.

5.1 Components of the Hepatic Microsomal Cytochrome P-450 System

5.1.1 Microsomal Protein Content

Comparison of Control and βNF-Treatment Groups.

The differences observed in the microsomal protein content (expressed as milligrams of microsomal protein per gram of liver) between the control and β-naphthoflavone treatment groups ranged from no difference in content to an increase of 90 percent with an average increase in microsomal protein of 50 percent for the avian species. No significant difference in the content of microsomal protein was seen in the male rat. Significant increases in the content of microsomal protein were detected in the βNF-treatment groups compared to the respective control groups for the goose (1.9-fold) and Pekin duck (1.6-fold) ($p < 0.01$) and for the chicken (1.6-fold), turkey (1.3-fold), and Khaki Campbell duck (1.4-fold) ($p < 0.05$).

Comparison of Avian Species.

The constitutive levels of microsomal protein per gram of liver were comparable among the avian species. The microsomal protein levels for the βNF-treatment groups were significantly greater in the Pekin duck ($p < 0.01$) and Khaki Campbell duck ($p < 0.05$) compared to the quail.

Table 5-1

Treatment Group Designations

CC	CHICKEN CONTROL
CT	CHICKEN TREATMENT
QC	QUAIL CONTROL
QT	QUAIL TREATMENT
GC	GOOSE CONTROL
GT	GOOSE TREATMENT
TC	TURKEY CONTROL
TT	TURKEY TREATMENT
PDC	PEKIN DUCK CONTROL
PDT	PEKIN DUCK TREATMENT
KDC	KHAKI CAMPBELL DUCK CONTROL
KDT	KHAKI CAMPBELL DUCK TREATMENT
MDC	MUSCOVY DUCK CONTROL
MDT	MUSCOVY DUCK TREATMENT
ANC	ANSERIFORMES CONTROL
ANT	ANSERIFORMES TREATMENT
GAC	GALLIFORMES CONTROL
GAT	GALLIFORMES TREATMENT
MRC	MALE RAT CONTROL
MRT	MALE RAT TREATMENT

Comparison of Avian Orders.

For the avian orders, the Anseriformes βNF-treatment group had significantly higher microsomal protein per gram of liver than the Anseriformes control group ($p < 0.001$) and the Galliformes βNF-treatment group ($p < 0.05$). There was no significant difference noted ($p < 0.05$) between the Galliformes control and treatment groups.

Comparison of Avian Species and Male Rat.

In a comparison of the avian and mammalian species, the average constitutive level of microsomal protein per gram of liver for the avian species was approximately 60 percent of that detected in the male rat. The male rat control group had significantly higher ($p < 0.001$) microsomal protein per gram of liver than the quail, chicken, goose and Muscovy duck control groups. For the βNF-treatment groups, the levels of microsomal protein in the avian species were comparable to the levels of the treated male rat. The only significant ($p < 0.05$) difference between the male rat and the avian species was with the quail.

The content of hepatic microsomal protein in the avian species and the rat are presented in Table 5-2.

Table 5-2

Content of Microsomal Protein in Avian and Rat Hepatic Microsomes

Treatment Group	mg of microsomal protein per gram of liver
CC	7 ± 2
CT	11 ± 4
QC	7 ± 4
QT	7 ± 3
GC	7 ± 2
GT	13 ± 3
TC	9 ± 3
TT	12 ± 3
PDC	9 ± 2
PDT	14 ± 3
KDC	9 ± 2
KDT	13 ± 3
MDC	6 ± 4
MDT	11 ± 6
ANC	8 ± 3
ANT	13 ± 4
GAC	8 ± 3
GAT	10 ± 4
MRC	14 ± 2
MRT	14 ± 2

(table continued)

Table 5-2

Content of Microsomal Protein in Avian and Rat Hepatic Microsomes

Significant Differences Between Control and βNF-Treatment Groups		
p < 0.05	**p < 0.01**	**p < 0.001**
CT > CC	-	-
GT > GC	GT > GC	-
TT > TC	-	-
PDT > PDC	PDT > PDC	-
KDT > KDC	-	-
Significant Differences Between Avian Species		
p < 0.05	**p < 0.01**	**p < 0.001**
KDT > QT*	-	-
PDT > QT *	PDT > QT*	-
Significant Differences Between Avian Orders		
p < 0.05	**p < 0.01**	**p < 0.001**
ANT > ANC	ANT > ANC	ANC > ANT
ANT > GAT	-	-
Significant Differences Between Avian Species and Male Rat		
p < 0.05	**p < 0.01**	**p < 0.001**
MRC > QC	MRC > QC	MRC > QC*
MRC > CC	MRC > CC	MRC > CC*
MRC > GC	MRC > GC	MRC > GC*
MRC > MDC	MRC > MDC	MRC > MDC*
MRT > QT*	-	-

*Denotes a significant difference for Tukey's test but not for Scheffe's test

5.1.2 Specific Content of Cytochrome P-450

Comparison of Control and βNF-Treatment Groups.

Within the avian species, significant increases detected in cytochrome P-450 content (nmoles of cytochrome P-450 per milligram of microsomal protein) of the βNF-treatment groups ranged from 1.7- to 3.2-fold with an average increase of 2.7 times the levels of P-450 detected in the respective control groups. The greatest increase in the specific content of cytochrome P-450 was seen in the Pekin duck (3.2-fold) treatment group ($p < 0.05$). Significant increases in the specific content of P-450 were also noted in the βNF-treatment groups for the goose (2.6-fold), Muscovy duck (2.2-fold), turkey (1.7-fold), and male rat (1.4-fold) ($p < 0.001$); and the chicken (1.8-fold) ($p < 0.01$) when compared to the respective control groups for each species.

Comparison of Avian Species.

The specific content of cytochrome P-450 was comparable among the control groups of the avian species. For the βNF-treatment groups, the specific content of cytochrome P-450 for the turkey was significantly higher ($p < 0.05$) than that of the Khaki Campbell duck and the chicken. No other significant differences in cytochrome P-450 content were noted.

Comparison of Avian Orders.

The Anseriformes βNF-treatment group ($p < 0.001$) and the Galliformes βNF-treatment group ($p < 0.01$) had significantly higher P-450 content than their respective control groups. There was no significant difference in the P-450 content between the Galliformes and Anseriformes for either the control or treatment groups.

Comparison of Avian Species and Male Rat.

There were no significant differences ($p < 0.05$) noted between the levels of cytochrome P-450 in the avian species and the male rat for either the control or βNF-treatment groups.

The specific content of cytochrome P-450 of the avian and rat hepatic microsomes are presented in Table 5-3.

5.1.3 NADH-Dependent Cytochrome *c* Reductase

Comparison Control and βNF-Treatment Groups.

There were no significant differences in the NADH-dependent cytochrome *c* reductase activity between the control and βNF-treatment groups of any of the species studied.

Comparison of Avian Species.

There were numerous significant differences noted in NADH-dependent cytochrome *c* reductase activity between the avian control groups and between the avian treatment

Table 5-3

Cytochrome P-450 Content of Avian and Rat Hepatic Microsomes

Treatment Group	nmoles of P-450 per mg of microsomal protein
CC	0.5 ± 0.2
CT	0.9 ± 0.3
QC	1.2 ± 1.4
QT	2.0 ± 1.4
GC	0.6 ± 0.2
GT	1.6 ± 0.3
TC	0.6 ± 0.1
TT	2.1 ± 0.5
PDC	0.5 ± 0.1
PDT	1.6 ± 0.7
KDC	0.5 ± 0.2
KDT	0.9 ± 0.5
MDC	0.6 ± 0.3
MDT	1.3 ± 0.2
ANC	0.5 ± 0.2
ANT	1.4 ± 0.5
GAC	0.8 ± 0.8
GAT	1.7 ± 1.0
MRC	0.9 ± 0.2
MRT	1.3 ± 0.1

(table continued)

Table 5-3

Cytochrome P-450 Content of Avian and Rat Hepatic Microsomes

Significant Differences Between Control and βNF-Treatment Groups		
p < 0.05	**p < 0.01**	**p < 0.001**
CT > CC	CT > CC	-
GT > GC	GT > GC	GT > GC
TT > TC	TT > TC	TT > TC
PDT > PDC	-	-
MDT > MDC	MDT > MDC	MDT > MDC
MRT > MRC	MRT > MRC	MRT > MRC
Significant Differences Between Avian Species		
p < 0.05	**p < 0.01**	**p < 0.001**
TT > KDT*	-	-
TT > CT*	-	-
Significant Differences Between Avian Orders		
p < 0.05	**p < 0.01**	**p < 0.001**
ANT > ANC	ANT > ANC	ANT > ANC
GAT > GAC	GAT > GAC	-
Significant Differences Between Avian Species and Male Rat		
p < 0.05	**p < 0.01**	**p < 0.001**
-	-	-

*Denotes a significant difference for Tukey's test but not for Scheffe's test

groups. The most significant differences in activity ($p < 0.001$) between the control groups included goose > turkey, goose > chicken, Khaki Campbell duck > turkey, and Khaki Campbell duck > chicken. For the treatment groups, the most significant differences ($P < 0.001$) included a greater reductase activity in the Khaki Campbell duck than in the turkey or chicken. Less significant differences ($p < 0.01$) included greater activity in the goose control group compared to the Muscovy control group, Pekin duck control group compared to the turkey and chicken control groups, the Muscovy treatment group compared to the chicken and turkey treatment groups, and ($p < 0.05$) Pekin duck treatment group compared to the turkey treatment group.

Comparison of Avian Orders.

There were no significant differences between the reductase activities of the control and βNF-treatment Anseriformes groups or between the control and treatment Galliformes groups ($p < 0.05$). In comparison of the orders, the NADH-dependent cytochrome *c* reductase activity in the Anseriformes was significantly ($p < 0.001$) greater ($\approx$2-fold) than that of the Galliformes for both the control groups and the treatment groups.

Comparison of Avian Species and Male Rat.

The average constitutive reductase activity in the avian species was comparable to that of the male rat. Comparison of the male rat versus the avian species indicated only one significant difference and that was between the goose and the male rat control

groups. The reductase activity of the goose control was 1.9 times greater than that of the male rat control. In the βNF-treatment groups, there were no significant differences in activity between the avian species and the rat.

The NADH-dependent cytochrome *c* reductase activities (expressed as nmoles of cytochrome *c* reduced per minute per mg of microsomal protein) for the avian and male rat hepatic microsome control and β-naphthoflavone-treatment groups are presented in Table 5-4.

5.1.4 NADPH-Dependent Cytochrome *c* Reductase

Comparison of Control and βNF-Treatment Groups.

There were no significant differences in NADPH-dependent cytochrome *c* reductase activity between the control and βNF-treatment groups for any of the species studied.

Comparison of Avian Species.

NADPH-dependent reductase activity was comparable among the avian control groups. For the βNF-treatment groups, the only significant difference ($p < 0.05$) noted between the avian species was a greater reductase activity in the goose compared to the Muscovy duck.

Table 5-4

NADH-Dependent Cytochrome *c* Reductase Activity in Avian and Rat Hepatic Microsomes

Treatment Group	nmoles of cytochrome *c* reduced per minute per mg of protein
CC	232 ± 98
CT	212 ± 95
QC	584 ± 299
QT	541 ± 135
GC	923 ± 247
GT	583 ± 302
TC	233 ± 85
TT	199 ± 47
PDC	641 ± 129
PDT	614 ± 780
KDC	759 ± 224
KDT	804 ± 391
MDC	461 ± 208
MDT	746 ± 283
ANC	696 ± 259
ANT	687 ± 296
GAC	349 ± 246
GAT	317 ± 188
MRC	488 ± 92
MRT	551 ± 85

(table continued)

Table 5-4

NADH-Dependent Cytochrome *c* Reductase Activity in Avian and Rat Hepatic Microsomes

Significant Differences Between Control and βNF-Treatment Groups		
p < 0.05	**p < 0.01**	**p < 0.001**
-	-	-
Significant Differences Between Avian Species		
p < 0.05	**p < 0.01**	**p < 0.001**
GC > MDC	GC > MDC*	-
GC > TC	GC > TC	GC > TC
GC > CC	GC > CC	GC > CC
KDC > TC	KDC > TC	KDC > TC*
KDC > CC	KDC > CC	KDC > CC*
PDC > TC*	PDC > TC*	-
PDC > CC*	PDC > CC*	-
KDT > CT	KDT > CT*	KDT > CT*
KDT > TT	KDT > TT*	KDT > TT*
MDT > CT	MDT > CT*	-
MDT > TT	MDT >TT*	-
PDT > TT*	-	-
Significant Differences Between Avian Orders		
p < 0.05	**p < 0.01**	**p < 0.001**
ANC > GAC	ANC > GAC	ANC > GAC
ANT > GAT	ANT > GAT	ANT > GAT
Significant Differences Between Avian Species and Male Rat		
p < 0.05	**p < 0.01**	**p < 0.001**
-	-	-

*Denotes a significant difference for Tukey's test but not for Scheffe's test

Comparison of Avian Orders.

There were no significant differences in reductase activity between the control and βNF-treatment groups for either the Galliformes or the Anseriformes. Likewise, there were no significant differences in reductase activity between the Galliformes and Anseriformes control or βNF-treatment groups.

Comparison of Avian Species and Male Rat.

Overall, NADPH-dependent cytochrome *c* reductase activity was comparable for all species studied for both the control and treatment groups. Therefore, there were no significant differences in activity between the avian species and the male rat.

The NADPH-dependent cytochrome *c* reductase activities (expressed as nmoles of cytochrome *c* reduced per minute per mg of microsomal protein) for the avian and male rat hepatic microsomal control and β-naphthoflavone-treatment groups are presented in Table 5-5.

5.2 Ethoxyresorufin-*O*-Deethylase

5.2.1 Ethoxyresorufin-*O*-Deethylase - Specific Activity

Comparison of Control and βNF-Treatment Groups.

Dramatically greater levels of EROD activity (based on specific activity) were observed in the βNF-treatment groups compared to the respective control groups for all species studied. The differences in activity level for each species, in order of

Table 5-5

NADPH-Cytochrome *c* Reductase Activity in Avian and Rat Hepatic Microsomes

Treatment Group	nmoles of cytochrome *c* reduced per minute per mg of protein
CC	93 ± 25
CT	109 ± 37
QC	165 ± 132
QT	162 ± 60
GC	160 ± 33
GT	176 ± 48
TC	117 ± 24
TT	110 ± 35
PDC	117 ± 25
PDT	123 ± 18
KDC	114 ± 13
KDT	121 ± 21
MDC	84 ± 14
MDT	98 ± 23
ANC	119 ± 35
ANT	129 ± 40
GAC	130 ± 80
GAT	126 ± 50
MRC	168 ± 32
MRT	137 ± 42

(table continued)

Table 5-5

NADPH-Cytochrome *c* Reductase Activity in Avian and Rat Hepatic Microsomes

Significant Differences Between Control and βNF-Treatment Groups		
p < 0.05	**p < 0.01**	**p < 0.001**
-	-	-
Significant Differences Between Avian Species		
p < 0.05	**p < 0.01**	**p < 0.001**
GT > MDT*	-	-
Significant Differences Between Avian Orders		
p < 0.05	**p < 0.01**	**p < 0.001**
-	-	-
Significant Differences Between Avian Species and Male Rat		
p < 0.05	**p < 0.01**	**p < 0.001**
-	-	-

*Denotes a significant difference for Tukey's test but not for Scheffe's test

decreasing magnitude, included: male rat (69-fold) > chicken (39-fold) > goose ≈ turkey ≈ Pekin duck (20-fold) > quail ≈ Khaki Campbell duck (16-fold) > Muscovy duck (9-fold). All differences in activity were significant at the $p < 0.001$ level except for the quail which was significant at the $p < 0.01$ level. Although the Muscovy duck had the smallest difference in activity between groups, it should be noted that this species demonstrated the highest constitutive level of EROD activity among the species studied (including the male rat) and that in the βNF-treatment

groups, the activity of the Muscovy was comparable to the activity of the other avian treatment groups.

Comparison of Avian Species.

Constitutive ethoxyresorufin-*O*-deethylase activity was comparable among the avian species studied. For the treatment groups, significant differences in activity included Pekin duck > quail ($p < 0.001$) and the turkey > quail ($p < 0.01$). Other differences of lesser significance ($p < 0.05$, differences significant for Tukey's test but not for Scheffe's test) between the avian treatment groups included greater EROD activity in the Khaki Campbell duck, chicken and Muscovy duck compared to that observed in the quail.

Comparison of Avian Orders.

The EROD activities of the Anseriformes and Galliformes βNF-treatment groups were significantly ($p < 0.001$) increased over the activities of their respective control groups. The Anseriformes and the Galliformes βNF-treatment groups demonstrated a 15-fold and 25-fold greater activity, respectively, than the constitutive activities of the respective control groups. Constitutive EROD activity of the Anseriformes was significantly ($p < 0.05$) higher than that of the Galliformes. No significant difference in activity was detected between the Anseriformes and Galliformes βNF-treatment groups.

Comparison of Avian Species and Male Rat.

The avian species had constitutive EROD activities comparable to those of the male rat. Therefore, there were no significant differences ($p < 0.05$) in EROD activity between the control groups of the avian species and the male rat. In contrast, the EROD activity demonstrated by the male rat treatment group was significantly greater ($p < 0.001$) than that seen in the treatment groups of each of the avian species studied. Statistical comparison of the activities of the control and βNF treatment groups indicated that the male rat had a 4-times greater (69-fold vs. 17-fold) increase in EROD activity than the average increase exhibited by the avian species as a whole.

The ethoxyresorufin-*O*-deethylase activities (expressed as pmoles of resorufin produced per minute per mg of microsomal protein) for the avian and male rat hepatic microsomal control and β-naphthoflavone-treatment groups are presented in Table 5-6.

5.2.2 Ethoxyresorufin-*O*-Deethylase - Molar Activity

Comparison of Control and βNF-Treatment Groups.

As with specific activity, significant differences in EROD activity were seen between the control and βNF-treatment groups for all the species studied. However, the increases noted in terms of turnover number were less pronounced than those noted in terms of specific activity. Differences in molar activity, between the control and βNF-treated groups, presented in order of decreasing magnitude, included male rat

Table 5-6

Ethoxyresorufin-*O*-Deethylase Activity in Avian and Rat Hepatic Microsomes - Specific Activity

Treatment Group	pmoles of resorufin produced per minute per mg of protein
CC	14 ± 7
CT	546 ± 205
QC	11 ± 7
QT	178 ± 92
GC	22 ± 11
GT	483 ± 137
TC	31 ± 28
TT	637 ± 122
PDC	34 ± 25
PDT	700 ± 254
KDC	35 ± 19
KDT	558 ± 124
MDC	64 ± 65
MDT	544 ± 149
ANC	39 ± 37
ANT	571 ± 181
GAC	18 ± 19
GAT	454 ± 247
MRC	25 ± 5
MRT	1719 ± 339

(table continued)

Table 5-6

Ethoxyresorufin-*O*-Deethylase Activity in Avian and Rat Hepatic Microsomes - Specific Activity

Significant Differences Between Control and βNF-Treatment Groups		
p < 0.05	**p < 0.01**	**p < 0.001**
CT > CC	CT > CC	CT > CC
QT > QC	QT > QC	-
GT > GC	GT > GC	GT > GC
TT > TC	TT > TC	TT > TC
PDT > PDC	PDT > PDC	PDT > PDC
KDT > KDC	KDT > KDC	KDT > KDC
MDT > MDC	MDT > MDC	MDT > MDC
MRT > MRC	MRT > MRC	MRT > MRC
Significant Differences Between Avian Species		
p < 0.05	**p < 0.01**	**p < 0.001**
PDT > QT	PDT > QT	PDT > QT*
TT > QT	TT > QT*	-
KDT > QT*	-	-
CT > QT *	-	-
MDT > QT *	-	-
Significant Differences Between Avian Orders		
p < 0.05	**p < 0.01**	**p < 0.001**
ANC > GAC	-	-
ANT > ANC	ANT > ANC	ANT > ANC
GAT > GAC	GAT > GAC	GAT > GAC

(table continued)

Table 5-6

Ethoxyresorufin-*O*-Deethylase Activity in Avian and Rat Hepatic Microsomes - Specific Activity

Significant Differences Between Avian Species and Male Rat		
$p < 0.05$	$p < 0.01$	$p < 0.001$
MRT > PDT	MRT > PDT	MRT > PDT
MRT > TT	MRT > TT	MRT > TT
MRT > KDT	MRT > KDT	MRT > KDT
MRT > CT	MRT > CT	MRT > CT
MRT > MDT	MRT > MDT	MRT > MDT
MRT > GT	MRT > GT	MRT > GT
MRT > QT	MRT > QT	MRT > QT

*Denotes a significant difference for Tukey's test but not for Scheffe's test

(47.5-fold) > chicken (21.4-fold) > Khaki Campbell duck (9.3-fold) > Pekin duck ≈ Japanese quail (7.8-fold) > goose ≈ turkey (7.2-fold) > Muscovy duck (3.7-fold). These differences were significant at the $p < 0.001$ level for the Muscovy duck and the male rat, at the $p < 0.01$ level for the turkey, quail, and chicken, and at the $p < 0.05$ level for the goose, Pekin duck, and Khaki Campbell duck. As discussed in terms of specific activity, the Muscovy duck had the highest constitutive level of EROD activity but had the smallest difference in EROD activity between the control and treatment groups based on turnover number. However, for the βNF

treatment group, EROD activity in the Muscovy duck was comparable to the levels observed by the treatment groups of the other species.

Comparison of Avian Species.

Only a few significant differences in EROD activity were noted between the avian species. For the control groups, EROD activity was comparable among the avian species except for a significant difference between the Muscovy duck and the quail ($p < 0.05$, the difference was significant for Tukey's test but not for Scheffe's test). Between the βNF-treatment groups, significant differences in EROD activity included greater activity in the Khaki Campbell duck ($p < 0.01$) and the chicken ($p < 0.05$, the difference was significant for Tukey's test but not for Scheffe's test) compared to the quail.

Comparison of Avian Orders.

The EROD activities were significantly ($p < 0.001$) greater in the Anseriformes (6.6-fold) and Galliformes (12-fold) βNF-treatment groups compared to the respective control groups. The activity of the Anseriformes control group was significantly ($p < 0.01$) greater (2.6-fold) than that of the Galliformes control group. However, there were no significant differences in EROD activity between the Anseriformes and Galliformes βNF-treatment groups.

Comparison of Avian Species and Male Rat.

The level of constitutive EROD activity for the avian species was equivalent to that of the male rat. In contrast, the βNF-treatment group for the male rat demonstrated EROD activities that were significantly greater (3-fold) than the activities, on average, of the βNF-treatment avian groups. Levels of significance for these differences included $p < 0.001$ for the Pekin duck, Muscovy duck, turkey, goose, and quail, $p < 0.01$ for the chicken, and $p < 0.05$ (difference was significant for Tukey's test but not for Scheffe's test) for the Khaki Campbell duck.

The ethoxyresorufin-*O*-deethylase activities (expressed as pmoles of resorufin produced per minute per nmole of cytochrome P-450) for the avian and male rat hepatic microsomal control and β-naphthoflavone-treatment groups are presented in Table 5-7.

5.3 Benzo[*a*]pyrene Hydroxylase

5.3.1 3-Hydroxybenzo[*a*]pyrene - Specific Activity

Comparison of Control and βNF-Treatment Groups.

Based on specific activity, significant increases in the formation of 3-hydroxybenzo[*a*]pyrene (3-OH B[*a*]P) were observed in the βNF-treatment groups, compared to the respective control groups, for the chicken (4-fold) ($p < 0.001$) and the quail (2-fold) ($p < 0.05$). No significant difference in 3-OH B[*a*]P formation was detected in the other species studied.

Table 5-7

Ethoxyresorufin-*O*-Deethylase Activity in Avian and Rat Hepatic Microsomes - Molar Activity

Treatment Group	pmoles of resorufin produced per minute per nmole of P-450
CC	30 ± 15
CT	644 ± 341
QC	13 ± 10
QT	102 ± 49
GC	44 ± 34
GT	310 ± 117
TC	46 ± 38
TT	330 ± 130
PDC	69 ± 49
PDT	544 ± 345
KDC	80 ± 61
KDT	747 ± 472
MDC	114 ± 114
MDT	424 ± 110
ANC	77 ± 71
ANT	506 ± 328
GAC	30 ± 27
GAT	359 ± 304
MRC	28 ± 2
MRT	1280 ± 224

(table continued)

Table 5-7

Ethoxyresorufin-*O*-Deethylase Activity in Avian and Rat Hepatic Microsomes - Molar Activity

Significant Differences Between Control and βNF-Treatment Groups		
p < 0.05	**p < 0.01**	**p < 0.001**
CT > CC	CT > CC	-
QT > QC	QT > QC	-
GT > GC	-	-
TT > TC	TT > TC	-
PDT > PDC	-	-
KDT > KDC	-	-
MDT > MDC	MDT > MDC	MDT > MDC
MRT > MRC	MRT > MRC	MRT > MRC
Significant Differences Between Avian Species		
p < 0.05	**p < 0.01**	**p < 0.001**
MDC > QC*	-	-
KDT > QT	KDT > QT*	-
CT > QT*	-	-
Significant Differences Between Avian Orders		
p < 0.05	**p < 0.01**	**p < 0.001**
ANT > ANC	ANT > ANC	ANT > ANC
ANC > GAC	ANC > GAC	-
GAT > GAC	GAT > GAC	GAT > GAC

(table continued)

Table 5-7

Ethoxyresorufin-*O*-Deethylase Activity in Avian and Rat Hepatic Microsomes - Molar Activity

Significant Differences Between Avian Species and Male Rat		
p < 0.05	**p < 0.01**	**p < 0.001**
MRT > KDT*	-	-
MRT > CT	MRT > CT*	-
MRT > PDT	MRT > PDT	MRT > PDT*
MRT > MDT	MRT > MDT	MRT > MDT
MRT > TT	MRT > TT	MRT > TT
MRT > GT	MRT > GT	MRT > GT
MRT > QT	MRT > QT	MRT > QT

*Denotes a significant difference for Tukey's test but not for Scheffe's test

Comparison of Avian Species.

Significant differences in the formation of 3-OH B[*a*]P between the avian control groups included turkey > chicken ($p < 0.001$) and quail ($p < 0.01$), and Khaki Campbell duck > chicken ($p < 0.01$). Less significant ($p < 0.05$, difference significant for Tukey's test but not significant for Scheffe's test) differences between control groups included turkey > Pekin duck and turkey > goose. It is interesting to note that the greatest production of 3-OH B[*a*]P was by the turkey for both the control and βNF-treated groups. In addition, the level of 3-OH B[*a*]P produced by the turkey control was greater than the levels produced by the treatment groups of the

other avian species and, in spite of the outstanding production of 3-OH B[*a*]P by the turkey, only a 13% increase in 3-OH B[*a*]P formation was present in the treatment group compared to the control. In contrast, the chicken had the lowest 3-OH B[*a*]P formation among the avian species, but had the greatest increase (4.3-fold) in formation between the control and βNF-treatment groups. In comparison of the avian βNF-treatment groups, the formation of 3-OH B[*a*]P by the turkey was significantly greater than that of the chicken. Less significant ($p < 0.05$, difference significant for Tukey's test but not significant for Scheffe's test) differences between the treatment groups included greater 3-OH B[*a*]P production by the turkey, Khaki Campbell duck and quail compared to the goose.

Comparison of Avian Orders.

The formation of 3-OH B[*a*]P by the Galliformes treatment group was significantly ($p < 0.05$) greater (2-fold) than that of the Galliformes control group. The Anseriformes control and treatment groups were not significantly different. Production of 3-OH B[*a*]P by the Galliformes treatment group was significantly ($p < 0.05$) greater than that of the Anseriformes treatment group but there was no significant difference detected between the control groups.

Comparison of Avian Species and Male Rat.

Significant differences in the production of 3-OH B[*a*]P between the avian and male rat controls included greater 3-OH B[*a*]P formation in the turkey (3.2-fold) ($p <$

0.01) and Khaki Campbell duck (2.7-fold) ($p < 0.05$, difference significant for Tukey's test but not significant for Scheffe's test) compared to that of the male rat. Formation of 3-OH B[*a*]P by the βNF-treatment groups was also significantly greater in the turkey (2.9-fold) ($p < 0.01$) and the Khaki Campbell duck (2.1-fold) ($p < 0.05$, difference significant for Tukey's test but not significant for Scheffe's test) than in the male rat.

The benzo[*a*]pyrene hydroxylase activities (expressed as pmoles of 3-OH B[*a*]P produced per minute per mg of microsomal protein) for the avian and male rat hepatic microsomal control and β-naphthoflavone-treatment groups are presented in Table 5-8.

5.3.2 3-Hydroxybenzo[*a*]pyrene - Molar Activity

Comparison of Control and Treatment Groups.

In comparison with the control groups, significantly ($p < 0.05$) greater quantities of 3-OH B[*a*]P were produced (based on turnover number) by the treatment groups of the chicken (2.4-fold) and quail (1.7-fold). In contrast, significantly ($p < 0.05$) lower levels of 3-OH B[*a*]P formation were produced by the treatment groups of the goose (4-fold), turkey (3-fold), and Muscovy duck (1.6-fold) respective to their control groups. No difference in 3-OH production was seen between the control and βNF-treatment groups of the Pekin duck, Khaki Campbell duck, and male rat.

Table 5-8

Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Formation of 3-Hydroxybenzo[*a*]pyrene - Specific Activity

Treatment Group	pmoles of 3-OH B[*a*]P produced per minute per mg of protein
CC	102 ± 51
CT	436 ± 97
QC	236 ± 264
QT	534 ± 184
GC	317 ± 174
GT	232 ± 80
TC	714 ± 256
TT	805 ± 244
PDC	323 ± 264
PDT	481 ± 93
KDC	595 ± 237
KDT	577 ± 139
MDC	381 ± 196
MDT	520 ± 133
ANC	404 ± 236
ANT	453 ± 172
GAC	351 ± 337
GAT	592 ± 237
MRC	221 ± 112
MRT	280 ± 130

(table continued)

Table 5-8

Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Formation of 3-Hydroxybenzo[*a*]pyrene - Specific Activity

Significant Differences Between Control and Treatment Groups		
p < 0.05	**p < 0.01**	**p < 0.001**
CC > CT	CC > CT	CC > CT
QT > QC	-	-
Significant Differences Between Avian Species		
p < 0.05	**p < 0.01**	**p < 0.001**
TC > PDC*	-	-
TC > GC*	-	-
TC > QC	TC > QC*	-
TC > CC	TC > CC	TC > CC*
KDC > CC	KDC > CC*	-
TT > PDT*	-	-
TT > CT*	TT > CT*	-
TT > GT*	-	-
KDT > GT*	-	-
QT > GT*	-	-
Significant Differences Between Avian Orders		
p < 0.05	**p < 0.01**	**p < 0.001**
GAT > ANT	-	-
GAT > GAC	-	-

(table continued)

Table 5-8

Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Formation of 3-Hydroxybenzo[*a*]pyrene - Specific Activity

Significant Differences Between Avian Species and Male Rat		
$p < 0.05$	**$p < 0.01$**	**$p < 0.001$**
TC > MRC	TC > MRC*	-
KDC > MRC*	-	-
TT > MRT	TT > MRT	TT > MRT
KDT > MRT*	-	-

*Denotes a significant difference for Tukey's test but not for Scheffe's test.

Comparison of Avian Species.

Significant differences between the avian control groups included greater 3-OH B[*a*]P formation by the Khaki Campbell duck compared to the chicken, quail ($p < 0.001$), and goose ($p < 0.05$, difference significant for Tukey's test but not significant for Scheffe's test) and by the turkey compared to the chicken and quail ($p < 0.001$). In terms of molar activity, the Khaki Campbell duck control and treatment groups had the greatest formation of 3-OH B[*a*]P rather than the turkey as was seen when the statistical analysis was based on specific activity.

Comparison of Avian Orders.

The Anseriformes control group produced a significantly ($p < 0.01$) greater quantity (2-fold) of 3-0H B[*a*]P than the treatment group. There was no significant difference in 3-OH B[*a*]P metabolite formation seen between the Galliformes control and βNF-treatment groups. In addition, no significant difference was detected between the avian orders for either the control or treatment groups.

Comparison of Avian Species and Male Rat.

Comparisons between the avian and male rat control groups indicated the turkey ($p < 0.001$) and the Khaki Campbell duck ($p < 0.01$) produced significantly greater quantities of 3-OH B[*a*]P than the male rat. Constitutive production of 3-OH B[*a*]P by the remaining species was comparable to that of the male rat. Of the treatment groups, the only species significantly different from the male rat were the Khaki Campbell duck ($p < 0.001$) and the chicken ($p < 0.05$, difference significant for Tukey's test but not significant for Scheffe's test). The Khaki Campbell duck and chicken treatment groups produced greater quantities of 3-OH B[*a*]P, 3.2- and 2.4-fold, respectively, than was produced by the male rat. Formation of the 3-OH B[*a*]P metabolite by the treatment groups of the other species was similar to that observed in the male rat.

The benzo[*a*]pyrene hydroxylase activities (expressed as pmoles of 3-OH B[*a*]P produced per nmole of cytochrome P-450) for the avian and male rat hepatic microsomal control and β-naphthoflavone-treatment groups are presented in Table 5-9.

5.3.3 9-Hydroxybenzo[*a*]pyrene - Specific Activity

Comparison of Control and βNF-Treatment Groups.

Evaluation of the results of the 6 individual animals within each control group indicated that 9-hydroxybenzo[*a*]pyrene (9-OH B[*a*]P) was produced by only 5 of the male rats, 5 of the Pekin ducks, and 4 of the Muscovy ducks. Six out of 6 animals produced 9-OH B[*a*]P within the control groups of the other species. Based on specific activity, the formation of 9-OH B[*a*]P in the βNF-treatment groups was significantly greater than that of the control groups for all the species studied. The greatest increases following βNF-treatment were present in the Pekin duck (4.4-fold) and the chicken (4-fold), followed by the male rat (3.3-fold), goose (3-fold), Khaki Campbell duck (3-fold), Muscovy duck (3-fold), quail (2-fold), and the turkey (1.5-fold). Levels of significance for these increases included $p < 0.001$ for the chicken, goose, Pekin duck, and Khaki Campbell duck and $p < 0.05$ for the quail, Muscovy, and male rat.

Table 5-9

Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Formation of 3-Hydroxybenzo[*a*]pyrene - Molar Activity

Treatment Group	pmoles of 3-OH B[*a*]P 7,8-produced per minute per nmole of P-450
CC	219 ± 113
CT	516 ± 221
QC	192 ± 58
QT	317 ± 106
GC	568 ± 333
GT	144 ± 46
TC	1201 ± 553
TT	422 ± 132
PDC	639 ± 426
PDT	339 ± 113
KDC	1293 ± 669
KDT	702 ± 276
MDC	663 ± 152
MDT	415 ± 154
ANC	791 ± 517
ANT	440 ± 262
GAC	537 ± 580
GAT	409 ± 175
MRC	245 ± 112
MRT	219 ± 89

(table continued)

Table 5-9

Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Formation of 3-Hydroxybenzo[*a*]pyrene - Molar Activity

Significant Differences Between Control and βNF-Treatment Groups		
p < 0.05	**p < 0.01**	**p < 0.001**
CT > CC	-	-
QT > QC	-	-
GT > GC	-	-
TC > TT	-	-
MDC > MDT	-	-
Significant Differences Between Avian Species		
p < 0.05	**p < 0.01**	**p < 0.001**
KDC > GC*	-	-
KDC > CC	KDC > CC	KDC > CC*
KDC > QC	KDC > QC	KDC > QC*
TC > CC	TC > CC*	TC > CC*
TC > QC	TC > QC*	TC > QC*
KDT > PDT*	KDT > PDT*	-
KDT > QT	KDT > QT*	-
KDT > GT	KDT > GT	KDT > GT
CT > GT*	CT > GT*	-
Significant Differences Between Avian Orders		
p < 0.05	**p < 0.01**	**p < 0.001**
ANC > ANT	ANC > ANT	-

(table continued)

Table 5-9

Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Formation of 3-Hydroxybenzo[*a*]pyrene - Molar Activity

Significant Differences Between Avian Species and the Male Rat		
$p < 0.05$	$p < 0.01$	$p < 0.001$
KDC > MRC	KDC > MRC	KDC > MRC*
TC > MRC	TC > MRC*	-
KDT > MRT	KDT > MRT	KDT > MRT
CT > MRT*	-	-

*Denotes a significant difference for Tukey's test but not for Scheffe's test

Comparison of Avian Species.

Ranking of the avian species based on the formation of 9-OH B[*a*]P resulted in following sequences: turkey > chicken ≈ quail ≈ goose ≈ Muscovy duck ≈ Khaki Campbell duck > Pekin duck for the control groups and turkey ≈ chicken > goose > quail > Pekin duck > Muscovy duck > Khaki Campbell duck for the treatment groups. It should be noted that the turkey had the greatest formation of 9-OH B[*a*]P among the control groups and among the treatment groups, yet had the smallestdifference (1.5-fold) in formation between the control and treatment groups. It is also interesting to note that the constitutive level of 9-OH formation for the turkey was greater (≈2-fold) than the treated levels of the other avian species (excluding the chicken). Numerous significant ($p < 0.001$) differences were noted between the avian species. For the control groups, significant ($p < 0.001$)

differences were noted between the turkey and each of the other avian species. Production of the 9-OH B[*a*]P metabolite was similar among the remaining avian control groups. Between the treatment groups, 9-OH B[*a*]P formation by the turkey and the chicken was significantly ($p < 0.001$) greater than formation by the goose, quail, Muscovy duck, Pekin duck, and Khaki Campbell duck. No significant differences were observed between the treatment groups of the other avian species.

Comparison of Avian Orders.

Significant differences were detected between the control and treatment groups of both the Anseriformes ($p < 0.001$) and the Galliformes ($p < 0.01$). Production of 9-OH B[*a*]P by the βNF-treatment groups were significantly greater (Anseriformes 3-fold, Galliformes, 2-fold) than that of the respective control groups. Significant differences between orders included greater formation of 9-OH B[*a*]P by the Galliformes control group (3.9-fold) compared to the Anseriformes control group ($p < 0.01$) and greater production in the Galliformes treatment group (2.4-fold) compared to the Anseriformes treatment group ($p < 0.001$).

Comparison of the Avian Species and Male Rat.

In general, 9-OH B[*a*]P production by the avian species equaled or exceeded that of the male rat for both the control groups and the treatment groups. Significant ($p < 0.001$) differences in 9-OH B[*a*]P formation were present between the turkey and the male rat control groups, chicken and male rat treatment groups and the turkey and the

male rat treatment groups. The differences in 9-OH B[*a*]P formation between the control and βNF-treatment groups for the avian species (1.5- to 4-fold) were comparable to that of the male rat (3-fold).

The benzo[*a*]pyrene hydroxylase activities (expressed as pmoles of 9-OH B[*a*]P produced per minute per mg of microsomal protein) for the avian and male rat hepatic microsomal control and β-naphthoflavone-treatment groups are presented in Table 5-10.

5.3.4 9-Hydroxybenzo[*a*]pyrene - Molar Activity

Comparison of Control and βNF-Treatment Groups.

The only significant ($p < 0.05$) difference in 9-OH B[*a*]P formation detected between the control and treatment groups, based on molar activity, was for the chicken (2-fold increase in the treatment group respective to the control group).

Comparison of Avian Species.

The levels of 9-OH B[*a*]P formation among the avian species included turkey > chicken ≈ Muscovy duck ≈ goose ≈ Khaki Campbell duck ≈ quail ≈ Pekin duck for the control groups and chicken > turkey ≈ Khaki Campbell duck ≈ Muscovy duck ≈ goose ≈ quail ≈ Pekin duck for the βNF-treatment groups. There were numerous significant differences in 9-OH formation between the avian species.

Table 5-10

Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Formation of 9-Hydroxybenzo[*a*]pyrene - Specific Activity

Treatment Group	pmoles of 9-OH B[a]P produced per minute per mg of protein
CC	49 ± 21
CT	195 ± 53
QC	41 ± 41
QT	78 ± 19
GC	28 ± 16
GT	83 ± 22
TC	154 ± 74
TT	232 ± 70
PDC	14 ± 8
PDT	61 ± 17
KDC	20 ± 9
KDT	59 ± 20
MDC	23 ± 29
MDT	73 ± 34
ANC	21 ± 17
ANT	69 ± 25
GAC	81 ± 71
GAT	168 ± 83
MRC	24 ± 14
MRT	78 ± 48

(table continued)

Table 5-10

Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Formation of 9-Hydroxybenzo[*a*]pyrene - Specific Activity

Significant Differences Between Control and βNF-Treatment Groups		
p < 0.05	**p < 0.01**	**p < 0.001**
CT > CC	CC > CT	CC > CT
QT > QC	-	-
GT > GC	GT > GC	GT > GC
PDT > PDC	PDT > PDC	PDT > PDC
KDT > KDC	KDT > KDC	KDT > KDC
MDT > MDC	-	-
MRT > MRC	-	-
Significant Differences Between Avian Species		
p < 0.05	**p < 0.01**	**p < 0.001**
TC > CC	TC > CC	TC > CC*
TC > QC	TC > QC	TC > QC
TC > GC	TC > GC	TC > GC
TC > MDC	TC > MDC	TC > MDC
TC > KDC	TC > KDC	TC > KDC
TC > PDC	TC > PDC	TC > PDC
TT > GT	TT > GT	TT > GT
TT > QT	TT > QT	TT > QT
TT > MDT	TT > MDT	TT > MDT
TT > PDT	TT > PDT	TT > PDT
TT > KDT	TT > KDT	TT > KDT
CT > GT	CT > GT*	CT > GT*

(table continued)

Table 5-10

Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Formation of 9-Hydroxybenzo[*a*]pyrene - Specific Activity

CT > QT	CT > QT	CT > QT*
CT > MDT	CT > MDT	CT > MDT*
CT > PDT	CT > PDT	CT > PDT
CT > KDT	CT > KDT	CT > KDT
Significant Differences Between Avian Orders		
$p < 0.05$	**$p < 0.01$**	**$p < 0.001$**
ANT > ANC	ANT > ANC	ANT > ANC
GAT > GAC	GAT > GAC	-
GAT > ANT	GAT > ANT	GAT > ANT
GAC > ANC	GAC > ANC	-
Significant Differences Between Avian Species and the Male Rat		
$p < 0.05$	**$p < 0.01$**	**$p < 0.001$**
TC > MRC	TC > MRC	TC > MRC
TT > MRT	TT > MRT	TT > MRT
CT > MRT	CT > MRT	CT > MRT*

*Denotes a significant difference for Tukey's test but not for Scheffe's test

Comparisons of the control groups indicated that 9-OH B[*a*]P formation by the turkey was significantly ($p < 0.001$) greater than that of the other avian species. For the treatment groups, 9-OH B[*a*]P formation by the chicken was significantly greater than

than that of the other avian treatment groups ($p < 0.01$ for the turkey and $p < 0.001$ for the other species).

Comparison of Avian Orders.

The Anseriformes treatment group produced a significantly ($p < 0.05$) greater level of 9-OH B[*a*]P than the control group. No significant difference was seen between the Galliformes control and treatment groups. 9-Hydroxybenzo[*a*]pyrene formation in the Galliformes control and treatment groups was significantly ($p < 0.01$) greater than the formation seen in the Anseriformes control and treatment groups, respectively.

Comparison of Avian Species and Male Rat.

In the avian species, the formation of 9-OH B[*a*]P by the control and treatment groups was equivalent to or greater than that of the male rat control. Significant ($p < 0.001$) differences between the avian species and the male rat included greater 9-OH B[*a*]P formation in the turkey control compared to the male rat control and in the chicken treatment group compared to the male rat treatment group.

The benzo[*a*]pyrene hydroxylase activities (expressed as pmoles of 9-OH B[*a*]P produced per nmole of cytochrome P-450) for the avian and male rat hepatic microsomal control and β-naphthoflavone-treatment groups are presented in Table 5-11.

Table 5-11
Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Formation of 9-Hydroxybenzo[*a*]pyrene - Molar Activity

Treatment Group	pmoles of 9-OH B[*a*]P produced per minute per nmole of P-450
CC	105 ± 38
CT	231 ± 103
QC	38 ± 11
QT	47 ± 18
GC	45 ± 20
GT	52 ± 15
TC	256 ± 142
TT	124 ± 50
PDC	29 ± 17
PDT	45 ± 18
KDC	42 ± 24
KDT	71 ± 27
MDC	51 ± 62
MDT	59 ± 35
ANC	38 ± 25
ANT	57 ± 26
GAC	132 ± 126
GAT	132 ± 100
MRC	28 ± 19
MRT	60 ± 35

(table continued)

Table 5-11
Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Formation of 9-Hydroxybenzo[*a*]pyrene - Molar Activity

Significant Differences Between Control and βNF-Treatment Groups		
p < 0.05	**p < 0.01**	**p < 0.001**
CC > CT	-	-
Significant Differences Between Avian Species		
p < 0.05	**p < 0.01**	**p < 0.001**
TC > CC	TC > CC*	TC > CC*
TC > GC	TC > GC	TC > GC
TC > KDC	TC > KDC	TC > KDC
TC > QC	TC > QC	TC > QC
TC > MDC	TC > MDC	TC > MDC
TC > PDC	TC > PDC	TC > PDC
CT > TT	CT > TT*	-
CT > KDT	CT > KDT	CT > KDT
CT > MDT	CT > MDT	CT > MDT
CT > GT	CT > GT	CT > GT
CT > QT	CT > QT	CT > QT
CT > PDT	CT > PDT	CT > PDT
Significant Differences Between Avian Orders		
p < 0.01	**p < 0.01**	**p < 0.001**
GAC > ANC	GAC > ANC	-
GAT > ANT	GAT > ANT -	-
ANT > ANC	-	-

(table continued)

Table 5-11
Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Formation of 9-Hydroxybenzo[*a*]pyrene - Molar Activity

Significant Differences Between Avian Species and the Male Rat		
$p < 0.05$	$p < 0.01$	$p < 0.001$
TC > MRC	TC > MRC	TC > MRC
CT > MRT	CT > MRT	CT > MRT

*Denotes a significant difference for Tukey's test but not for Scheffe's test.

5.3.5 Benzo[*a*]pyrene 7,8-Dihydrodiol - Specific Activity

Comparison of Control and βNF-Treatment Groups.

Significant increases in benzo[*a*]pyrene 7,8-dihydrodiol (B[*a*]P 7,8-diol) formation were present in treatment groups, compared to the respective control groups, for all of the species studied. The most significant increases were seen in the chicken (22-fold), turkey (10-fold), Pekin duck (7-fold), and male rat (3-fold) ($p < 0.001$) followed by the Khaki Campbell duck (7-fold) ($p < 0.01$), quail (9-fold) and the goose ($p < 0.05$). It should be noted that B[*a*]P 7,8-diol was not produced by any member of the goose control group. In addition, B[*a*]P 7,8-diol was not produced by 2 animals within the Muscovy control group. However, all 6 animals within the goose and Muscovy duck treatment groups did produce B[*a*]P 7,8-diol.

Comparison of Avian Species.

Within the avian control groups, and within the avian treatment groups, the greatest production of B[*a*]P 7,8-diol was by the Muscovy duck. In comparison of the avian control groups, the only significant ($p < 0.05$, difference significant for Tukey's test but not significant for Scheffe's test) difference in B[*a*]P 7,8-diol formation was between the Muscovy duck and goose. In contrast, numerous significant differences were detected between the avian βNF-treatment groups. The most significant differences in B[*a*]P 7,8-diol formation included Muscovy > quail and goose ($p < 0.001$) followed by Khaki Campbell duck > quail and goose ($p < 0.01$). Less significant ($p < 0.05$, difference significant for Tukey's test but not significant for Scheffe's test) differences included the Muscovy > chicken and turkey and the Pekin duck > quail and goose.

Comparison of Avian Orders.

Both the Anseriformes and Galliformes treatment groups produced significantly ($p < 0.001$) greater (5-fold and 11-fold, respectively) levels of B[*a*]P 7,8-diol than their respective control groups. There was also a significantly greater formation of 7,8-diol by the Anseriformes control (4-fold) ($p < 0.05$) and treatment (2-fold) ($p < 0.01$) groups compared to the respective Galliformes control and treatment groups.

Comparison of Avian Species and Male Rat.

In general, the formation of B[*a*]P 7,8-diol by the avian control and treatment groups was equivalent to that of the male rat control and treatment groups, respectively. Therefore, there were no significant differences in B[*a*]P 7,8-diol formation between the avian species and the male rat for either the control or treatment groups. However, the difference in formation of B[*a*]P 7,8-diol between the control and βNF-treatment groups was much more pronounced (7 to 21.5-fold) in the avian species than in the male rat (3.3-fold) except for the Muscovy duck (3.3-fold).

The benzo[*a*]pyrene hydroxylase activities (expressed as pmoles of B[*a*]P 7,8-diol produced per minute per mg of microsomal protein) for the avian and male rat hepatic microsomal control and β-naphthoflavone-treatment groups are presented in Table 5-12.

5.3.6 Benzo[*a*]pyrene 7,8-Dihydrodiol - Molar Activity

Comparison of Control and βNF-Treatment Groups.

In terms of molar activity, B[*a*]P 7,8-diol formation by the βNF-treatment groups was significantly greater than that for the control groups for the quail, Khaki Campbell duck ($p < 0.001$), chicken, goose, turkey ($p < 0.01$), and male rat ($p < 0.05$). The most pronounced difference was seen in the quail (48-fold), followed by the chicken (13-fold), turkey (3-fold), and Khaki Campbell duck (3.3-fold).

Table 5-12

Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Formation of Benzo[*a*]pyrene 7,8-dihydrodiol Specific Activity

Treatment Group	pmoles of B[*a*]P 7,8-dihydrodiol produced per minute per mg of protein
CC	2 ± 2
CT	43 ± 14
QC	2 ± 4
QT	18 ± 12
GC	ND[1]
GT	17 ± 9
TC	4 ± 5
TT	40 ± 9
PDC	10 ± 7
PDT	66 ± 22
KDC	11 ± 9
KDT	76 ± 39
MDC	27 ± 38
MDT	89 ± 36
ANC	12 ± 21
ANT	61 ± 38
GAC	3 ± 4
GAT	34 ± 16
MRC	14 ± 11
MRT	46 ± 16

(table continued)

Table 5-12

Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Formation of Benzo[*a*]pyrene 7,8-dihydrodiol Specific Activity

Significant Differences Between Control and βNF-Treatment Groups		
p < 0.05	**p < 0.01**	**p < 0.001**
CT > CC	CT > CC	CT > CC
QT > QC	-	-
GT > GC	-	-
TT > TC	TT > TC	TT > TC
PDT > PDC	PDT > PDC	PDT > PDC
KDT > KDC	KDT > KDC	-
MDT > MDC	-	-
MRT > MRC	MRT > MRC	MRT > MRC
Significant Differences Between Avian Species		
p < 0.05	**p < 0.01**	**p < 0.001**
MDC > GC*	-	-
MDT > CT*	-	-
MDT > TT*	-	-
MDT > QT	MDT > QT	MDT > QT*
MDT > GT	MDT > GT	MDT > GT*
KDT > QT	KDT > QT*	-
KDT > GT	KDT > GT*	-
PDT > QT*	-	-
PDT > GT*	-	-

(table continued)

Table 5-12

Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Formation of Benzo[*a*]pyrene 7,8-dihydrodiol Specific Activity

Significant Differences Between Avian Orders		
$p < 0.05$	$p < 0.01$	$p < 0.001$
ANT > ANC	ANT > ANC	ANT > ANC
GAT > GAC	GAT > GAC	GAT > GAC
ANT > GAT	ANT > GAT	-
ANC > GAC	-	-
Significant Differences Between Avian Species and Male Rat		
$p < 0.05$	$p < 0.01$	$p < 0.001$
-	-	-

[1]ND indicates a nondetectable value
*Denotes a significant difference for Tukey's test but not for Scheffe's test

Comparison of Avian Species.

Between the avian control groups, there was a significantly greater formation of B[*a*]P 7,8-diol in the Muscovy compared to the goose and the quail ($p < 0.05$, difference significant for Tukey's test but not significant for Scheffe's test). Numerous differences were noted between the avian treatment groups. The most significant differences in B[*a*]P 7,8-diol formation for the βNF-treatment groups were between the Khaki Campbell duck and the turkey, quail, and goose ($p < 0.001$) and between the Muscovy and the turkey ($p < 0.01$), quail and goose ($p < 0.001$), and between

the Muscovy and turkey ($p < 0.01$). Less significant differences ($p < 0.05$, difference significant for Tukey's test but not significant for Scheffe's test) were present between the chicken and the quail, the chicken and the goose, the Pekin duck and the quail, and the Pekin duck and the goose.

Comparison of Avian Orders.

Both the Anseriformes ($p < 0.01$) and Galliformes ($p < 0.001$) treatment groups produced significantly greater (2-fold and 7-fold, respectively) levels of B[*a*]P 7,8-diol than their respective control groups. There was also a significantly ($p < 0.01$) greater production of B[*a*]P 7,8-diol metabolite by the Anseriformes control (6-fold) and treatment (2-fold) groups over the respective Galliformes groups.

Comparison of Avian Species and Male Rat.

In general, the avian control and treatment groups produced levels of B[*a*]P 7,8-diol comparable to that of the male rat. The only significant ($p < 0.01$) difference in B[*a*]P 7,8-diol formation between the avian and male rat groups was between the treatment groups of the Khaki Campbell duck and the male rat.

The benzo[*a*]pyrene hydroxylase activities (expressed as pmoles of B[*a*]P 7,8-diol produced per nmole of cytochrome P-450) for the avian and male rat hepatic microsomal control and β-naphthoflavone-treatment groups are presented in Table 5-13.

Table 5-13

Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Formation of Benzo[*a*]pyrene 7,8-dihydrodiol Molar Activity

Treatment Group	pmoles of B[*a*]P 7,8-dihydrodiol produced per minute per nmole of P-450
CC	4 ± 6
CT	51 ± 23
QC	0.4 ± 1
QT	19 ± 5
GC	ND[1]
GT	10 ± 5
TC	6 ± 7
TT	20 ± 8
PDC	23 ± 16
PDT	51 ± 25
KDC	25 ± 25
KDT	82 ± 20
MDC	42 ± 50
MDT	69 ± 38
ANC	23 ± 32
ANT	53 ± 36
GAC	4 ± 5
GAT	27 ± 23
MRC	14 ± 11
MRT	33 ± 13

(table continued)

Table 5-13

Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Formation of Benzo[*a*]pyrene 7,8-dihydrodiol Molar Activity

Significant Differences Between Control and βNF-Treatment Groups		
p < 0.05	**p < 0.01**	**p < 0.001**
CC > CT	CC > CT	-
QT > QC	QT > QC	QT > QC
GT > GC	GT > GC	-
TC > TT	TC > TT	-
KDC > KDT	KDC > KDT	KDC > KDT
MRT > MRC	-	-
Significant Differences Between Avian Species		
p < 0.05	**p < 0.01**	**p < 0.001**
MDC > QC*	-	-
MDC > GC*	-	-
KDT > TT	KDT> TT	KDT > TT*
KDT > GT	KDT > GT	KDT > GT
KDT > QT	KDT > QT	KDT > QT
MDT > TT	MDT > TT*	-
MDT > GT	MDT > GT	MDT > GT*
MDT > QT	MDT > QT	MDT > QT*
CT > GT*	-	-
CT > QT*	-	-
PDT > GT*	-	-
PDT > QT*	-	-

(table continued)

Table 5-13

Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Formation of Benzo[*a*]pyrene 7,8-dihydrodiol Molar Activity

Significant Differences Between Avian Orders		
p < 0.05	**p < 0.01**	**p < 0.001**
ANC > GAC	ANC > GAC	-
ANT > GAT	ANT > GAT	-
ANT > ANC	ANT > ANC	-
GAT > GAC	GAT > GAC	GAT > GAC
Comparison of Avian Species and Male Rat		
p < 0.05	**p < 0.01**	**p < 0.001**
KDT > MRT	KDT > MRT*	-

[1]ND indicates a nondetectable value
*Denotes a significant difference for Tukey's test but not for Scheffe's test.

5.3.7 Benzo[*a*]pyrene 9,10-Dihydrodiol - Specific Activity

Comparison of Control and βNF-Treatment Groups.

Of the control groups, the only species which formed the B[*a*]P 9,10-dihydrodiol (B[*a*]P 9,10-diol) metabolite were the Khaki Campbell duck and the male rat. Within the Khaki Campbell duck control group, only 2 of the 6 animals formed B[*a*]P 9,10-diol. In the βNF-treatment groups, B[*a*]P 9,10-diol was formed by all the species studied. However, only 20 to 40 percent of the animals within each of the avian

treatment groups formed the metabolite. The only significant difference between control and treatment groups for this metabolite was for the goose ($p < 0.05$).

Comparison of Avian Species.

Metabolite formation among the avian species was comparable for both the control and treatment groups. Hence, there were no significant differences between the control groups nor between the βNF-treatment groups of the avian species.

Comparison of Avian Orders.

Benzo[*a*]pyrene 9,10-dihydrodiol formation was significantly greater in the β-treatment groups compared to the respective control groups for both the Anseriformes ($p < 0.001$) and the Galliformes ($p < 0.05$). It should be noted that within the control groups of the Galliformes order, none of the species formed B[*a*]P 9,10-diol and within the Anseriformes order, only the Khaki Campbell duck formed the metabolite. There were no significant differences in B[*a*]P 9,10-diol formation between the Anseriformes and Galliformes.

Comparison of Avian Species and Male Rat.

Benzo[*a*]pyrene 9,10-dihydrodiol formation by the male rat control and treatment groups was significantly ($p < 0.001$) greater than that of the respective avian groups.

The benzo[*a*]pyrene hydroxylase activities (expressed as pmoles of B[*a*]P 9,10-diol formed per minute per mg of microsomal protein) in avian and male rat hepatic microsomes of control and β-naphthoflavone treatment groups are presented below in Table 5-14.

5.3.8 Benzo[*a*]pyrene 9,10-Dihydrodiol - Molar Activity

Comparison of Control and βNF-Treatment Groups.

In terms of molar activity, the only species that had a significant difference in B[*a*]P 9,10-diol formation between the control and βNF-treatment groups were the goose and the male rat. Comparable quantities of B[*a*]P 9,10-diol were formed by the control and treatment groups of the remaining avian species.

Comparison of Avian Species.

Benzo[*a*]pyrene 9,10-dihydrodiol production was similar among the control groups and among the treatment groups of the avian species. There were no significant differences in B[*a*]P 9,10-diol formation ($p < 0.05$) between the control groups nor between the βNF-treatment groups of the avian species.

Comparison of Avian Orders.

Benzo[*a*]pyrene 9,10-dihydrodiol formation by the Galliformes βNF-treatment group was significantly ($p < 0.05$) greater than that of the control group. No other significant differences were noted within or between the orders.

Table 5-14

Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Formation of Benzo[*a*]pyrene 9,10-dihydrodiol - Specific Activity

Treatment Group	pmoles of B[*a*]P 9,10-dihydrodiol produced per minute per mg of protein
CC	ND[1]
CT	25 ± 24
QC	ND
QT	10 ± 22
GC	ND
GT	25 ± 25
TC	ND
TT	4 ± 8
PDC	ND
PDT	20 ± 20
KDC	7 ± 14
KDT	23 ± 21
MDC	ND
MDT	13 ± 16
ANC	2 ± 7
ANT	20 ± 20
GAC	ND
GAT	13 ± 20
MRC	96 ± 29
MRT	204 ± 51

(table continued)

Table 5-14

Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Formation of Benzo[*a*]pyrene 9,10-dihydrodiol - Specific Activity

Significant Differences Between Control and βNF-Treatment Groups		
p < 0.05	**p < 0.01**	**p < 0.001**
GT > GC	-	-
Significant Differences Between Avian Species		
p < 0.05	**p < 0.01**	**p < 0.001**
-	-	-
Significant Differences Between Avian Orders		
p < 0.05	**p < 0.01**	**p < 0.001**
ANT > ANC	ANT > ANC	ANT > ANC
GAT > GAC	-	-
Significant Differences Between Avian Species and Male Rat		
p < 0.05	**p < 0.01**	**p < 0.001**
MRC > KDC	MRC > KDC	MRC > KDC
MRC > TC	MRC > TC	MRC > TC
MRC > CC	MRC > CC	MRC > CC
MRC > PDC	MRC > PDC	MRC > PDC
MRC > MDC	MRC > MDC	MRC > MDC
MRC > QC	MRC > QC	MRC > QC
MRC > GC	MRC > GC	MRC > GC
MRT > GT	MRT > GT	MRT > GT
MRT > CT	MRT > CT	MRT > CT
MRT > KDT	MRT > KDT	MRT > KDT
MRT > PDT	MRT > PDT	MRT > PDT

(table continued)

Table 5-14

Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Formation of Benzo[*a*]pyrene 9,10-dihydrodiol - Specific Activity

MRT > MDT	MRT > MDT	MRT > MDT
MRT > QT	MRT > QT	MRT > QT
MRT > TT	MRT > TT	MRT > TT

[1]ND indicates a nondetectable value

Comparison of Avian Species and Male Rat.

In terms of molar activity, significant differences in the formation of B[*a*]P 9,10-diol between the avian species and the male rat were identical to those based on specific activity as discussed in Section 5.3.7.

The benzo[*a*]pyrene hydroxylase activities (in terms of molar activity - pmoles of B[*a*]P 9,10-diol formed per minute per nmoles of cytochrome P-450) in avian and male rat hepatic microsomal of control and β-naphthoflavone treatment groups are presented in Table 5-15.

5.3.9 Total Metabolite Formation - Specific Activity

Total metabolite formation is represented by the sum of the four B[*a*]P metabolites identified and quantitated in this study: 3-hydroxybenzo[*a*]pyrene, 9-

hydroxybenzo[*a*]pyrene, benzo[*a*]pyrene 7,8-dihydrodiol, and benzo[*a*]pyrene 9,10-dihydrodiol.

Comparison of Control and βNF-Treatment Groups.

Significant increases in total B[*a*]P metabolite formation by the treatment groups, compared to the respective control groups, were seen for the chicken (5-fold) ($p < 0.001$), quail (2-fold), Pekin duck (2-fold), and male rat (1.7-fold) ($p < 0.05$). For the turkey, Khaki Campbell duck, Muscovy duck, and goose, no significant differences in total B[*a*]P metabolite formation were observed between the control and treatment groups.

Comparison of Avian Species.

The most significant ($p < 0.001$) differences in total metabolite formation between the avian species were present between the turkey and chicken control groups and the turkey and goose treatment groups. Significant ($p < 0.01$) differences were also detected between the turkey and the Pekin duck, goose, and quail control groups. Less significant differences ($p < 0.05$, difference significant for Tukey's test but not significant for Scheffe's test) were seen between the turkey and Muscovy duck control groups, the Khaki Campbell duck and chicken control groups, the turkey and quail treatment groups, and the turkey and Pekin duck treatment groups. The rankings of total B[*a*]P metabolite formation for the avian control groups included turkey > Muscovy duck ≈ Pekin duck ≈ goose ≈ quail ≈ chicken and Khaki

Table 5-15

Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Formation of Benzo[*a*]pyrene 9,10-dihydrodiol - Molar Activity

Treatment Group	pmoles of B[*a*]P 9,10-dihydrodiol produced per minute per nmole of P-450
CC	ND[1]
CT	32 ± 31
QC	ND
QT	3 ± 5
GC	ND
GT	14 ± 13
TC	ND
TT	2 ± 4
PDC	ND
PDT	14 ± 12
KDC	27 ± 58
KDT	20 ± 15
MDC	ND
MDT	10 ± 14
ANC	7 ± 28
ANT	14 ± 14
GAC	ND
GAT	12 ± 23
MRC	110 ± 29
MRT	151 ± 32

(table continued)

Table 5-15

Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Formation of Benzo[*a*]pyrene 9,10-dihydrodiol - Molar Activity

Significant Differences Between Control and βNF-Treatment Groups		
p < 0.05	**p < 0.01**	**p < 0.001**
GT > GC	-	-
MRT > MRC	-	-
Significant Differences Between Avian Species		
p < 0.05	**p < 0.01**	**p < 0.001**
-	-	-
Significant Differences Between Avian Orders		
p < 0.05	**p < 0.01**	**p < 0.001**
GAT > GAC	-	-
Significant Differences Between Avian Species and Male Rat		
p < 0.05	**p < 0.01**	**p < 0.001**
MRC > KDC	MRC > KDC	MRC > KDC
MRC > TC	MRC > TC	MRC > TC
MRC > CC	MRC > CC	MRC > CC
MRC > PDC	MRC > PDC	MRC > PDC
MRC > MDC	MRC > MDC	MRC > MDC
MRC > QC	MRC > QC	MRC > QC
MRC > GC	MRC > GC	MRC > GC
MRT > KDT	MRT > KDT	MRT > KDT
MRT > TT	MRT > TT	MRT > TT
MRT > CT	MRT > CT	MRT > CT
MRT > PDT	MRT > PDT	MRT > PDT

(table continued)

Table 5-15

Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Formation of Benzo[*a*]pyrene 9,10-dihydrodiol - Molar Activity

MRT > MDT	MRT > MDT	MRT > MDT
MRT > QT	MRT > QT	MRT > QT
MRT > GT	MRT > GT	MRT >GT

[1]ND indicates a nondetectable value

Campbell duck > chicken. For the avian treatment groups, the ranking of formation included turkey > chicken ≈ quail ≈ goose. It is interesting to note, that among the avian species, the turkey produced the greatest quantity of total B[*a*]P metabolites for both the control and treatment groups but had the smallest difference in metabolite formation between the control and treatment groups.

Comparison of Avian Orders.

There was a significant increase in total B[*a*]P metabolite formation in the treatment groups compared to the control groups for both the Anseriformes ($p < 0.05$) and Galliformes ($p < 0.01$). While there was no significant difference between the control groups, metabolite formation by the Galliformes treatment group was significantly ($p < 0.05$) greater than that of the Anseriformes treatment group.

Comparison of Avian Species and Male Rat.

Comparison of total B[*a*]P metabolite formation between the avian species and male rat indicated that all of the avian species, with the exception of the turkey, produced total metabolite levels that were equivalent to those of the male rat. The only significant differences in total B[*a*]P metabolite formation between the avian species and the male rat were for the turkey and male rat control groups ($p < 0.01$) and for the turkey and male rat treatment groups ($p < 0.05$, difference significant for Tukey's test but not significant for Scheffe's test). Metabolite formation by the avian treatment groups was 2-fold to 5-fold greater than that of the respective control groups. For the male rat a 1.7-fold greater metabolite production was seen in the treatment group compared to the control group.

The benzo[*a*]pyrene hydroxylase activities (expressed as pmoles of total metabolites formed per minute per mg of microsomal protein) in avian and male rat hepatic microsomal control and β-naphthoflavone treatment groups are presented in Table 5-16.

5.3.10 Total Metabolite Formation - Molar Activity

Comparison of Control and βNF-Treatment Groups.

Based on molar activity, a significantly ($p < 0.05$) greater level of total B[*a*]P metabolites were formed by the chicken (2.5-fold) and quail (1.7-fold) treatment groups compared to their respective control groups. Significant ($p < 0.05$) decreases

Table 5-16

Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Total Metabolite[1] Formation - Specific Activity

Treatment Group	pmoles of total B[*a*]P metabolites produced per minute per mg of protein
CC	153 ± 73
CT	699 ± 176
QC	278 ± 308
QT	640 ± 233
GC	345 ± 183
GT	358 ± 125
TC	872 ± 324
TT	1081 ± 309
PDC	347 ± 264
PDT	628 ± 128
KDC	634 ± 254
KDT	735 ± 210
MDC	431 ± 248
MDT	690 ± 216
ANC	439 ± 254
ANT	603 ± 222
GAC	434 ± 406
GAT	806 ± 306
MRC	353 ± 159
MRT	608 ± 208

(table continued)

Table 5-16

Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Total Metabolite[1] Formation - Specific Activity

Significant Differences Between Control and βNF-Treatment Groups		
p < 0.05	**p < 0.01**	**p < 0.001**
CT > CC	CT > CC	CT > CC
QT > QC	-	-
PDT > PDC	-	-
MRT > MRC	-	-
Significant Differences Between Avian Species		
p < 0.05	**p < 0.01**	**p < 0.001**
TC > MDC*	-	-
TC > PDC*	TC > PDC*	-
TC > GC*	TC > GC*	-
TC > QC	TC > QC*	-
TC > CC	TC > CC	TC > CC*
KDC > CC*	-	-
TT > QT*	-	-
TT > GT	TT > GT	TT > GT*
TT > PDT*	-	-
Significant Differences Between Avian Orders		
p < 0.05	**p < 0.01**	**p < 0.001**
GAT > ANT	-	-
ANT > ANC	-	-
GAT > GAC	GAT > GAC	-

(table continued)

Table 5-16

Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Total Metabolite[1] Formation - Specific Activity

Significant Differences Between Avian Species and Male Rat		
$p < 0.05$	**$p < 0.01$**	**$p < 0.001$**
TC > MRC*	TC > MRC*	-
TT > MRT*	-	-

[1]Total metabolite formation includes the metabolites B[*a*]P 9,10-dihydrodiol, B[*a*]P 7,8-dihydrodiol, 9-hydroxy-B[*a*]P, and 3-hydroxy-B[*a*]P
*Denotes a significant difference for Tukey's test but not for Scheffe's test.

in total metabolite formation were detected in the treatment groups of the goose (2.8-fold) and turkey (2.7-fold) relative to their control groups. No significant differences were seen between the control and treatment groups of the remaining avian species.

Comparison of Avian Species.

Comparison of the avian control groups indicated significant differences in the metabolism of B[*a*]P between the turkey and the chicken ($p < 0.001$), the turkey and the quail ($p < 0.001$), the Khaki Campbell duck and the quail ($p < 0.001$) and the Khaki Campbell duck and the chicken ($p < 0.01$). Significant differences in total metabolite formation between the treatment groups included the Khaki Campbell duck and the goose ($p < 0.001$), the chicken and the goose ($p < 0.001$), the Khaki Campbell duck and the quail ($p < 0.01$), the Khaki Campbell duck and the Pekin

duck ($p < 0.05$, difference significant for Tukey's test but not significant for Scheffe's test), and the chicken and the quail ($p < 0.05$, difference significant for Tukey's test but not significant for Scheffe's test).

Comparison of Avian Orders.

A significantly ($p < 0.05$) lower level of total metabolite formation was present in the Anseriformes treatment group (1.6-fold) compared to the control group. No significant difference was observed in the Galliformes treatment group respective to the control group. No significant differences between the orders were noted.

Comparison of Avian Species and Male Rat.

Comparison of total metabolite formation in the avian controls and the male rat control indicated that total formation by the turkey and the Khaki Campbell duck control groups were significantly ($p < 0.01$) greater (3.7- and 3.5-fold, respectively) than that of the male rat control group. Total metabolite formation in the avian treatment groups was comparable to that of the male rat treatment group for all the species studied.

The benzo[*a*]pyrene hydroxylase activities (expressed as pmoles of total metabolite formed per minute per nmole of cytochrome P-450) in avian and male rat hepatic microsomal control and β-naphthoflavone treatment groups are presented in Table 5-17.

Table 5-17

Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Total Metabolite Formation - Molar Activity

Treatment Group	pmoles of total B[*a*]P metabolites produced per minute per nmole of P-450
CC	327 ± 149
CT	830 ± 369
QC	229 ± 68
QT	386 ± 99
GC	613 ± 348
GT	222 ± 70
TC	1463 ± 716
TT	542 ± 200
PDC	692 ± 444
PDT	445 ± 155
KDC	1387 ± 793
KDT	876 ± 306
MDC	741 ± 209
MDT	554 ± 248
ANC	858 ± 561
ANT	542 ± 313
GAC	672 ± 701
GAT	580 ± 305
MRC	396 ± 167
MRT	453 ± 148

(table continued)

Table 5-17

Benzo[*a*]pyrene Hydroxylase Activity in Avian and Rat Hepatic Microsomes: Total Metabolite Formation - Molar Activity

Significant Differences Between Control and βNF-Treatment Groups		
p < 0.05	**p < 0.01**	**p < 0.001**
TC > TT	-	-
CT > CC	-	-
QT > QC	-	-
GC > GT	-	-
Significant Differences Between Avian Species		
p < 0.05	**p < 0.01**	**p < 0.001**
TC > CC	TC > CC*	TC > CC*
TC > QC	TC > QC	TC > QC*
KDC > CC	KDC > CC*	-
KDC > QC	KDC > QC*	KDC > QC*
KDT > PDT*	-	-
KDT > QT*	KDT > QT*	-
KDT > GT	KDT > GT	KDT > GT*
CT > QT*	-	-
CT > GT	CT > GT*	CT > GT*
Significant Differences Between Avian Orders		
p < 0.05	**p < 0.01**	**p < 0.001**
ANC > ANT	-	-
Significant Differences Between Avian Species and Male Rat		
p < 0.05	**p < 0.01**	**p < 0.001**
TC > MRC	TC > MRC*	-
KDC > MRC*	KDC > MRC*	-

[1]Total metabolite formation includes the metabolites B[*a*]P 9,10-dihydrodiol, B[*a*]P 7,8-dihydrodiol, 9-hydroxy-B[*a*]P, and 3-hydroxy-B[*a*]P

*Denotes a significant difference for Tukey's test but not for Scheffe's test.

5.3.11 Overview of Benzo[*a*]pyrene Metabolism

This section provides an overview of the hepatic microsomal oxidative metabolism of B[*a*]P by each species and each avian order. The B[*a*]P metabolite profiles produced by each species and each order are represented by a ranking of the metabolites in order of decreasing magnitude of formation; 3-hydroxybenzo[*a*]pyrene is designated by 3-OH, 9-hydroxybenzo[*a*]pyrene is designated by 9-OH, benzo[*a*]pyrene 7,8-dihydrodiol is designated by 7,8-diol, and benzo[*a*]pyrene 9,10-dihydrodiol is designated by 9,10-diol. The changes observed in the metabolic profiles associated with βNF-treatment are also summarized. In addition, shifts in the profiles due to relative changes in metabolite formation in the treatment group compared to the control group are presented as the percent change observed for each metabolite based on the percent each metabolite contributed to the total metabolite formation.

Chicken.

The metabolic profiles for the chicken were as follows:

Control: 3-OH > 9-OH > 7,8-diol

Treatment: 3-OH > 9-OH > 7,8-diol > 9,10-diol

Changes in the profile associated with βNF-treatment included:

- A significant increase in the formation of 3-OH, 4.3-fold (specific activity) and 2.4-fold (molar activity)
- A significant increase in the formation of 9-OH, 4-fold (specific activity) and 2.2 fold (molar activity)

- A significant increase in the formation of 7,8-diol, 22-fold (specific activity) and 12.8-fold (molar activity)
- Formation of 9,10-diol (no significant increase)
- In terms of specific activity, a relative increase in the formation of 9,10-diol (3.6%) and 7,8-diol (4.9%) and a relative decrease in the formation of 9-OH (4.1%) and 3-OH (4.4%)
- In terms of molar activity, a relative increase in the formation of 9,10-diol (3.9%) and 7,8-diol (4.9%) and a relative decrease in the formation of 9-OH (4.2%) and 3-OH (4.6%)
- A significant increase in B[*a*]P hydroxylase activity (based on total metabolite formation)

Quail.

The metabolic profiles for the quail were as follows:

Control: 3-OH > 9-OH > 7,8-diol

Treatment: 3-OH > 9-OH > 7,8-diol > 9,10-diol

Changes in the profile associated with βNF-treatment included:

- A significant increase in the formation of 3-OH, 2.3-fold (specific activity) and 1.7-fold (molar activity)
- A significant increase in the formation of 9-OH (1.9-fold) based on specific activity and no change based on molar activity
- A significant increase in the formation of 7,8-diol, 9-fold (specific activity) and 47.5-fold (molar activity)
- Formation of 9,10-diol (no significant increase)
- In terms of specific activity, a relative increase in the formation of 9,10-diol (1.6%) and 7,8-diol (2.1%) and a relative decrease in the formation of 9-OH (2.5%) and 3-OH (1.2%)

- In terms of molar activity, a relative increase in the formation of 9,10-diol (0.8%) and 7,8-diol (4.7%) and a relative decrease in the formation of 9-OH (4.3%) and 3-OH (1.2%)

- A significant increase in B[*a*]P hydroxylase activity (based on total metabolite formation)

Goose.

The metabolic profiles for the goose were as follows:

Control: 3-OH > 9-OH

Treatment: 3-OH > 9-OH > 9,10-diol ≈ 7,8-diol

Changes in the profile associated with βNF-treatment included:

- A significant decrease in the formation of 3-OH, 3.9-fold based on molar activity and no significant change based on specific activity

- A significant increase in the formation of 9-OH, 3-fold based on specific activity and no change based on molar activity

- Formation of 7,8-diol (increase significant)

- Formation of 9,10-diol (increase significant)

- In terms of specific activity, a relative increase in the formation of 9,10-diol (7%), 7,8-diol (4.8%), and 9-OH (15.1%) and a relative decrease in the formation of 3-OH (26.9%)

- In terms of molar activity, a relative increase in the formation of 9,10-diol (6.4%), 7,8-diol (4.5%), and 9-OH (16.3%) and a relative decrease in the formation of 3-OH (27.2%)

- A significant decrease in B[*a*]P hydroxylase activity in terms of molar activity, no change in terms of specific activity (based on total metabolite formation)

Turkey.

The metabolic profiles for the turkey were as follows:

Control: 3-OH > 9-OH > 7,8-diol

Treatment: 3-OH > 9-OH > 7,8-diol > 9,10-diol

Changes in the metabolic profile associated with βNF-treatment included:

- A significant decrease (2.8-fold) in 3-OH formation in terms of molar activity, no significant change in terms of specific activity
- No significant change in 9-OH formation
- A significant increase in 7,8-diol formation, 10-fold (specific activity) and 3.3-fold (molar activity)
- Formation of 9,10-diol (increase not significant)
- In terms of specific activity, a relative increase in the formation of 9,10-diol (0.3%), 7,8-diol (3.2%), and 9-OH (3.8%) and a relative decrease in the formation of 3-OH (7.4%)
- In terms of molar activity, a relative increase in the formation of 9,10-diol (0.4%), 7,8-diol (3.1%), and 9-OH (4.3%) and a relative decrease in the formation of 3-OH (7.8%)
- A significant decrease in B[*a*]P hydroxylase activity in terms of molar activity, no change in terms of specific activity (based on total metabolite formation)

Pekin Duck.

The metabolic profiles for the Pekin duck were as follows:

Control: 3-OH > 9-OH ≈ 7,8-diol

Treatment: 3-OH > 7,8-diol ≈ 9-OH > 9,10-diol

Changes in the metabolic profile associated with βNF-treatment included:

- No significant change in 3-OH formation

- A significant increase (4.4-fold) in 9-OH formation in terms of specific activity and no significant change in terms of molar activity
- A significant increase in 7,8-diol formation, 6.6-fold based on specific activity and no significant change based on molar activity
- Formation of 9,10-diol (increase not significant)
- In terms of specific activity, a relative increase in the formation of 9,10-diol (3.2%), 7,8-diol (7.6%), and 9-OH (9.3%) and a relative decrease in the formation of 3-OH (16.5%)
- In terms of molar activity, a relative increase in the formation of 9,10-diol (3.1%), 7,8-diol (8.1%), and 9-OH (5.8%) and a relative decrease in the formation of 3-OH (7.8%)
- A significant increase in B[*a*]P hydroxylase activity in terms of specific activity, no change in terms of molar activity (based on total metabolite formation)

Khaki Campbell Duck.

The metabolic profiles for the Khaki Campbell duck included:

Control: 3-OH > 9-OH ≈ 7,8-diol ≈ 9,10-diol

Treatment: 3-OH > 7,8-diol ≈ 9-OH > 9,10-diol

Changes in the metabolic profile associated with βNF-treatment included:

- No significant change in 3-OH formation
- A significant increase (3-fold) in 9-OH formation in terms of specific activity and no significant change in terms of molar activity
- A significant increase in 7,8-diol formation, 6.9-fold (specific activity) and 3.3-fold (molar activity)
- No significant change in the formation of 9,10-diol
- In terms of specific activity, a relative increase in the formation of 9,10-diol (2%), 7,8-diol (8.6%), and 9-OH (4.8%) and a relative decrease in the formation of 3-OH (15.5%)

- In terms of molar activity, a relative increase in the formation of 9,10-diol (0.3%), 7,8-diol (7.6%), and 9-OH (5.1%) and a relative decrease in the formation of 3-OH (13%)

Muscovy Duck.

The metabolic profiles for the Muscovy duck included:

Control: 3-OH > 7,8-diol ≈ 9-OH

Treatment: 3-OH > 7,8-diol ≈ 9-OH > 9,10-diol

Changes in the metabolic profile associated with βNF-treatment included:

- A significant decrease in the formation of 3-OH, 1.6-fold (molar activity) and no significant change based on specific activity
- A significant increase in the formation of 9-OH, 3.2-fold (specific activity) and no change based on molar activity
- A significant increase in the formation of 7,8-diol, 3.3-fold (specific activity) and 1.6-fold (molar activity)
- Formation of 9,10-diol (increase not significant)
- In terms of specific activity, a relative increase in the formation of 9,10-diol (1.9%), 7,8-diol (6.5%), and 9-OH (5.2%) and a relative decrease in the formation of 3-OH (13.6%)
- In terms of molar activity, a relative increase in the formation of 9,10-diol (1.9%), 7,8-diol (6.9%), and 9-OH (4%) and a relative decrease in the formation of 3-OH (27.2%)

Anseriformes.

The metabolic profiles for the Anseriformes were as follows:

Control: 3-OH > 9-OH ≈ 7,8-diol > 9,10-diol

Treatment: 3-OH > 9-OH ≈ 7,8-diol > 9,10-diol

Changes in the profile associated with βNF-treatment included:

- A significant decrease in the formation of 3-OH, 1.8-fold (molar activity) and no significant change based on specific activity
- A significant increase in the formation of 9-OH, 3.3-fold (specific activity) and 1.5 fold (molar activity)
- A significant increase in the formation of 7,8-diol, 5.1-fold (specific activity) and 2.3-fold (molar activity)
- A significant increase in the formation of 9,10-diol, 10-fold based on specific activity and no significant change based on molar activity
- In terms of specific activity, a relative increase in the formation of 9,10-diol (2.8%), 7,8-diol (7.4%), and 9-OH (6.6%) and a relative decrease in the formation of 3-OH (16.9%)
- In terms of molar activity, a relative increase in the formation of 9,10-diol (1.7%), 7,8-diol (6.7%), 9-OH (5.7%) and a relative decrease in the formation of 3-OH (14.1%)
- A significant increase in B[*a*]P hydroxylase activity (based on total metabolite formation)

Galliformes.

The metabolic profiles for the Galliformes were as follows:

Control: 3-OH > 9-OH > 7,8-diol

Treatment: 3-OH > 9-OH > 7,8-diol > 9,10-diol

Changes in the profile associated with βNF-treatment included:

- A significant decrease in the formation of 3-OH, 1.7-fold (specific activity) and no significant change based on molar activity
- A significant increase in the formation of 9-OH, 2.1-fold (specific activity) and no significant change based on molar activity
- A significant increase in the formation of 7,8-diol, 11.3-fold (specific activity) and 6.8-fold (molar activity)
- Formation of 9,10-diol (increase significant)

- In terms of specific activity, a relative increase in the formation of 9,10-diol (1.6%), 7,8-diol (3.5%), and 9-OH (2.2%) and a relative decrease in the formation of 3-OH (7.4%)

- In terms of molar activity, a relative increase in the formation of 9,10-diol (2.1), 7,8-diol (4%), 9-OH (3.1%) and a relative decrease in the formation of 3-OH (9.3%)

- A significant increase in B[*a*]P hydroxylase activity in terms of specific activity, no change in terms of molar activity (based on total metabolite formation)

Male Rat.

The metabolic profiles for the male rat were as follows:

Control: 3-OH > 9,10-diol > 9-OH > 7,8-diol

Treatment: 3-OH > 9,10-diol > 9-OH > 7,8-diol

Changes in the profile associated with βNF-treatment included:

- No significant changes in the formation of 3-OH

- A significant increase in the formation of 9-OH, 3.3-fold (specific activity) and no significant change in terms of molar activity

- A significant increase in the formation of 7,8-diol, 3.3-fold (specific activity) and 2.3-fold (molar activity)

- A significant increase in the formation of 9,10-diol, 1.4-fold based on molar activity, no significant change based on specific activity

- In terms of specific activity, a relative increase in the formation of 9,10-diol (6.6), 7,8-diol (3.7%), and 9-OH (6%) and a relative decrease in the formation of 3-OH (16.2%)

- In terms of molar activity, a relative increase in the formation of 9,10-diol (4.9%), 7,8-diol (3.6%), 9-OH (6%) and a relative decrease in the formation of 3-OH (14.4%)

- A significant increase in B[*a*]P hydroxylase activity in terms of specific activity , no change in terms of molar activity (based on total metabolite formation)

The metabolic profiles for the control and treatment groups of each species and avian order are presented in Table 5-18. The percent contribution of each metabolite to the total metabolite formation (based on the four metabolites quantitated in this study) is presented in Table 5-19 in terms of specific activity and in Table 5-20 in terms of molar activity.

Table 5-18

Metabolism of Benzo[*a*]pyrene by Avian and Rat Hepatic Microsomes Ranking of Metabolite Formation

CC	3-OH[1] > 9-OH[2] > 7,8-diol[3]
CT	3-OH > 9-OH > 7,8-diol > 9,10-diol[4]
QC	3-OH > 9-OH > 7,8-diol
QT	3-OH > 9-OH > 7,8-diol > 9,10-diol
GC	3-OH > 9-OH
GT	3-OH > 9-OH > 9,10-diol ≈ 7,8-diol
TC	3-OH > 9-OH > 7,8-diol
TT	3-OH > 9-OH > 7,8-diol > 9,10-diol
PDC	3-OH > 9-OH ≈ 7,8-diol
PDT	3-OH > 7,8-diol ≈ 9-OH > 9,10-diol
KDC	3-OH > 9-OH ≈ 7,8-diol ≈ 9,10-diol
KDT	3-OH > 7,8-diol ≈ 9-OH > 9,10-diol
MDC	3-OH > 7,8-diol ≈ 9-OH
MDT	3-OH > 7,8-diol ≈ 9-OH > 9,10-diol
ANC	3-OH > 9-OH ≈ 7,8-diol > 9,10-diol
ANT	3-OH > 9-OH ≈ 7,8-diol > 9,10-diol
GAC	3-OH > 9-OH > 7,8-diol
GAT	3-OH > 9-OH > 7,8-diol > 9,10-diol
MRC	3-OH > 9,10-diol > 9-OH > 7,8-diol
MRT	3-OH > 9,10-diol > 9-OH > 7,8-diol

[1]3-Hydroxybenzo[*a*]pyrene
[2]9-Hydroxybenzo[*a*]pyrene
[3]Benzo[*a*]pyrene 7,8-dihydrodiol
[4]Benzo[*a*]pyrene 9,10-dihydrodiol

Table 5-19

Metabolism of Benzo[*a*]pyrene by Avian and Rat Hepatic Microsomes Percent of Total Metabolites[1] Formed - Specific Activity

Treatment Group	3-OH[2]	9-OH[3]	7,8-Diol[4]	9,10-Diol[5]
CC	66.7	32.0	1.3	0
CT	62.3	27.9	6.2	3.6
QC	84.6	14.7	0.7	0
QT	83.4	12.2	2.8	1.6
GC	91.9	8.1	0	0
GT	65	23.2	4.8	7
TC	81.9	17.7	0.5	0
TT	74.5	21.5	3.7	0.3
PDC	93.1	4.0	2.9	0
PDT	76.6	9.7	10.5	3.2
KDC	94.0	3.2	1.7	1.1
KDT	78.5	8.0	10.3	3.1
MDC	88.4	5.3	6.3	0
MDT	74.8	10.5	12.8	1.9
ANC	92.0	4.8	2.7	0.5
ANT	75.1	11.4	10.1	3.3
GAC	80.7	18.6	0.7	0
GAT	73.3	20.8	4.2	1.6
MRC	62.2	6.8	3.9	27.0
MRT	46.0	12.8	7.6	33.6

[1]Total Metabolites refers to the four metabolites quantitated in this study
[2]3-Hydroxybenzo[*a*]pyrene
[3]9-Hydroxybenzo[*a*]pyrene
[4]Benzo[*a*]pyrene 7,8-dihydrodiol
[5]Benzo[*a*]pyrene 9,10-dihydrodiol

Table 5-20

Metabolism of Benzo[*a*]pyrene by Avian and Rat Hepatic Microsomes
Percent of Total Metabolites[1] Formed - Molar Activity

Treatment Group	3-OH[2]	9-OH[3]	7,8-Diol[4]	9,10-Diol[5]
CC	66.8	32.0	1.2	0
CT	62.2	27.8	6.1	3.9
QC	83.3	16.5	0.2	0
QT	82.1	12.2	4.9	0.8
GC	92.7	7.3	0	0
GT	65.5	23.6	4.5	6.4
TC	82.1	17.5	0.4	0
TT	74.3	21.8	3.5	0.4
PDC	92.5	4.2	3.3	0
PDT	75.5	10.0	11.4	3.1
KDC	93.2	3.0	1.8	2.0
KDT	80.2	8.1	9.4	2.3
MDC	87.7	6.7	5.6	0
MDT	75.0	10.7	12.5	1.9
ANC	92.1	4.4	2.7	0.8
ANT	78.0	10.1	9.4	2.5
GAC	79.8	19.6	0.6	0
GAT	70.5	22.7	4.6	2.1
MRC	61.7	7.0	3.5	27.7
MRT	47.3	13.0	7.1	32.6

[1]Total Metabolites refers to the four metabolites quantitated in this study
[2]3-Hydroxybenzo[*a*]pyrene
[3]9-Hydroxybenzo[*a*]pyrene
[4]Benzo[*a*]pyrene 7,8-dihydrodiol
[5]Benzo[*a*]pyrene 9,10-dihydrodiol

6.0 DISCUSSION OF RESULTS

The following sections 6.1 to 6.3 are a discussion of the results reported in Section 5.1 to 5.3.

6.1 Components of the Hepatic Cytochrome P-450 Monooxygenase System

6.1.1 Microsomal Protein and Cytochrome P-450 Content

6.1.1.1 Galliformes

This study demonstrated a yield of 7 mg microsomal protein/g liver for both the chicken and the quail. A review of the literature revealed that the reported yield of hepatic microsomal protein ranged from 4.3 to 19.8 mg protein/g liver for the chicken (Banton, 1990; Rinsky and Perry, 1983; Dalvi, *et al.*, 1987; Manning, *et al.*, 1990; Buynitzky, *et al.*, 1978; Buckpitt and Boyd, 1982; Riviere, *et al.*, 1985; Davison and Sell, 1972; Sifri, *et al.*, 1975) and from 4.8 to 31.2 mg/g for the quail (Carpenter, *et al.*, 1985; Buckpitt and Boyd, 1982; Riviere, *et al.*, 1983; Sifri, *et al.*, 1975; Riviere, *et al.*, 1985; Dalvi, *et al.*, 1987). These ranges represent data collected from birds of varying breed, age and sex. For the turkey, a single value of 6.5 $\pm$ 0.4 mg/g (Dalvi, *et al.*, 1987) was found in the literature which was comparable to the yield of 9 $\pm$ 3 mg/g observed in this study.

In the present study, the hepatic microsomal cytochrome P-450 levels observed for the chicken and quail were 0.5 and 1.2 nmoles/mg microsomal protein, respectively. Reported values ranged from 0.12 to 0.48 nmoles cytochrome P-450/mg microsomal protein for the chicken (Banton, 1989; Rinsky and Perry, 1983; Poland and Glover,

1977; Haug, *et al.*, 1980; Sell, *et al.*, 1971; Buckpitt and Boyd, 1982; Erich and Larsen, 1983; Manning, *et al.*, 1990; Buynitzky *et al.*, 1978; Riviere, *et al.*, 1985; Lapadula, *et al.*, 1984) and from 0.1 to 1.1 nmoles/mg for the quail (Carpenter, *et al.*, 1985; Buckpitt and Boyd, 1982; Riviere, *et al.*, 1983; Riviere, *et al.*, 1985; Dalvi, *et al.*, 1987). Only one study was found in the literature in which the hepatic P-450 content was characterized for the turkey (Dalvi *et al.*, 1987). These investigators observed a cytochrome P-450 content of 0.36 ± 0.03 nmoles/mg which is slightly lower than the 0.6 ± 0.1 nmoles/mg measured in this study.

The constitutive levels of microsomal protein and cytochrome P-450 were similar among the Galliformes species studied. Riviere *et al.* (1985) found similar yields of hepatic microsomal protein from the chicken and Japanese quail. Sifri and coworkers (1975) demonstrated a higher level of microsomal protein in the Japanese quail than in the White Leghorn chicken but observed comparable levels of P-450 for these species. Dalvi *et al.* (1987) found significantly ($p < 0.05$) greater constitutive levels of microsomal protein in the quail (Bobwhite) compared to the chicken (Rock-type) and turkey (white) but no significant difference was noted between the chicken and turkey. Quantitation of hepatic microsomal cytochrome P-450 content in the same study indicated significantly greater ($p < 0.05$) levels in the turkey compared to the chicken and quail and in the chicken compared to the quail (Dalvi, *et al.*, 1987).

Following βNF treatment, comparable levels of hepatic microsomal protein and cytochrome P-450 were observed among the Galliformes birds with the exception of a significantly ($p < 0.05$, significant for Tukey's test but not for Scheffe's test) greater (1.3 times) content of P-450 in the turkey compared to the chicken. The only notable difference seen between the species of this order was a significant induction of microsomal protein and P-450 content following βNF administration in the chicken and turkey but not in the quail. In the present study, microsomal protein yield in the chicken and turkey was 1.6- and 1.3-fold, respectively, the basal levels. For cytochrome P-450 content, a 1.8-fold increase was observed in the chicken and a 3.5-fold increase was seen in the turkey which was the largest increase observed among all the species examined. Two studies were found in the literature in which the yield of microsomal protein was determined for control and βNF- or 3MC-treated chickens. Buynitzky *et al.* (1978) demonstrated an increase amounting to 1.1- to 1.6-fold that of controls for microsomal protein following treatment with 3MC while no change was observed by Manning *et al.* (1990) following βNF treatment. Previous studies on the effects of βNF or 3MC administration on hepatic microsomal cytochrome P-450 content in the chicken demonstrated increases in P-450 content ranging from 1.7- to 4-fold (Banton, 1990; Haug *et al.*, 1980; Manning *et al.*, 1990; Powis, *et al.*, 1976; Buynitzky, *et al.*, 1978; Althaus, *et al.*, 1972; Darbey, *et al.*, 1984; Darbey *et al.*, 1985; Darbey, *et al.*, 1986; Topp and van Bladeren, 1986). Past studies on the hepatic cytochrome P-450 system of the Japanese quail found no change in the yield of microsomal protein associated with βNF or 3MC treatment while increases

ranging from 280 to 473% control values were reported for P-450 content (Buckpitt and Boyd, 1982; Carpenter, *et al.*, 1985; Buckpitt, *et al.*, 1982; Riviere, 1984). Neal *et al.*, (1986) observed a 176% increase in protein and a 905% increase in P-450 content of hepatic microsomes isolated from Japanese quail treated with 3MC. No studies were available which evaluated the response of the turkey hepatic microsomal system to βNF or other 3MC-type inducers.

6.1.1.2 Anseriformes

Reported levels of microsomal protein in untreated ducks (mallard) of varying ages and sex ranged from 13.8 to 31.6 mg/g liver (Davison and Sell, 1972; Gorsline *et al.*, 1981; Sifri, *et al.*, 1975; Dalvi, *et al.*, 1987). In comparison with these values, the levels determined in the present study were lower, 9 $\pm$ 2 mg/g for the Pekin and Khaki Campbell ducks and 6 $\pm$ 4 mg/g for the Muscovy duck. Only one study was found in the literature in which the components of the hepatic microsomal system of the goose (gray) were quantitated (Dalvi et al., 1987). These researchers found a basal microsomal protein level of 10 mg/g in this species which is consistent with the findings of this study.

Constitutive cytochrome P-450 levels observed in the present study for the Pekin, Khaki Campbell and Muscovy ducks are consistent with levels previously reported for other breeds of ducks. The cytochrome P-450 levels quantitated for hepatic microsomes isolated from mallard and Aylsebury x Pekin ducks ranged from 0.18 to

0.53 nmole/mg (Bartlett and Kirinya, 1976; Sifri, et al., 1975; Dalvi, et al., 1987). Only one study in the literature reported hepatic microsomal cytochrome P-450 content for the goose (Dalvi, *et al.*, 1987). These investigators observed a cytochrome P-450 level of 0.29 $\pm$ 0.04 nmoles/mg in the gray goose which is lower than the level observed in this study (0.6 $\pm$ 0.2 nmoles/mg).

This investigation found that the hepatic microsomal protein yield and cytochrome P-450 content was similar among the Anseriformes species examined for both the untreated and βNF treated birds. Dalvi *et al.* (1987) conducted a comparative study of these parameters for the gray goose and mallard duck. These researchers observed a significantly ($p < 0.05$) greater (1.6-fold) yield of hepatic microsomal protein in the mallard duck compared to the goose and a significantly ($p < 0.05$) greater (1.6-fold) content of cytochrome P-450 in the goose compared to the duck. No other studies were found in which the cytochrome P-450 content of the duck and goose were compared.

Induction of hepatic microsomal protein yield and cytochrome P-450 content was observed in the Anseriformes following treatment with βNF. However, it is interesting to note that for microsomal protein, all species showed an increase (1.4 to 1.9-fold) except for the Muscovy, whereas for P-450 content, increases (2.2 to 3.2-fold) were seen in all but the Khaki Campbell duck. No studies could be found in the literature which compared the hepatic microsomal protein and cytochrome P-450

content of control and βNF- (or 3MC) treated microsomes of any of the Anseriformes species examined here. However, one study did report a 320 percent increase in cytochrome P-450 content in the Eider duck (*Somateria mollissima*) associated with exposure to 3,3',4,4' tetrachlorobiphenyl, a 3MC-type inducer (Brouwer, 1991). The Eider duck is a sea duck and is classified within the Mergini tribe of the Anatidae family within the Anseriformes order (Kear, 1985).

6.1.1.3 Comparison of Galliformes and Anseriformes

The constitutive hepatic microsomal protein and cytochrome P-450 levels observed in the present study were comparable among the species of the Galliformes and Anseriformes orders. However, following βNF treatment, some differences were noted between the orders. The Anseriformes order demonstrated a 1.6-fold increase in protein in response to βNF treatment whereas no change in protein yield was observed in the Galliformes order. Consequently, a significantly ($p < 0.05$) greater (1.3-fold) level of microsomal protein was seen in the βNF treated Anseriformes compared to the βNF treated Galliformes. Induction of hepatic cytochrome P-450 content by βNF treatment was observed in both orders. However, the increase seen in the Anseriformes (3-fold) was slightly greater than that seen in the Galliformes (2-fold). No studies were found in the literature which compared microsomal protein levels for the Anseriformes and Galliformes species. Only one study was found in the literature which sought a comparison in cytochrome P-450 content between species of the Galliformes and Anseriformes orders. Sifri, Sell and Davison (1975) reported a

lower hepatic cytochrome P-450 level in the Mallard duck compared to that of the chicken and Japanese quail.

In general, comparison of these orders indicated similar levels of hepatic microsomal protein and cytochrome P-450 for control and treatment groups of the represented species.

6.1.1.4 Comparison of the Rat and the Avian Species

The present study demonstrated the rat hepatic microsomal protein and cytochrome P-450 levels, and the response of these parameters to βNF administration, to be consistent with findings reported by other researchers. These results indicated that the yield of microsomal protein in the rat was significantly greater than that of the quail, chicken, goose and Muscovy duck but was not significantly different than that of the turkey, Pekin duck or Khaki Campbell duck. One study, which examined the components of the cytochrome P-450 system of a variety of species, found that the yield of microsomal protein per gram of liver was comparable for the chicken, Khaki Campbell duck and rat (Patterson and Roberts, 1970). Dalvi *et al.*, (1987) found that the observed yield of microsomal protein for the rat was significantly greater (1.5- to 3.7-times) than that of the white turkey, chicken, gray goose and mallard duck but was comparable to that of the Bobwhite quail. The same group found a 1.5- to 3.3-fold greater cytochrome P-450 content in rat microsomes compared to microsomes isolated from the white turkey, Rock-type chicken, gray goose, mallard duck and

Bobwhite quail. Bartlett and Kirinya (1976) observed a 400% greater cytochrome P-450 level in the rat compared to the duck (Aylesbury x Pekin). In contrast to the findings of Dalvi *et al.* and Bartlett and Kirinya, the present study found no significant difference in the cytochrome P-450 content of the hepatic microsomes isolated from the rat and the avian species used in this study.

In general, the induction of microsomal protein and cytochrome P-450 content associated with βNF treatment was more marked in the avian species compared to the rat. Following βNF treatment, no significant changes in microsomal protein yield were observed for the rat, quail, or Muscovy duck. In contrast, significant increases (ranging from 1.3 to 1.9-fold) in protein yield were observed for the chicken, turkey, goose, Pekin and Khaki Campbell ducks. Topp and van Bladeren (1986) observed comparable levels of hepatic cytochrome P-450 in the chicken and the rat following βNF treatment as did this study. Induction of cytochrome P-450 content in the avian species (excluding the Japanese quail and Khaki Campbell duck) was approximately twice (2.7-fold greater than basal levels on average) the level of induction observed for the rat (1.4-fold greater than constitutive levels). This finding is consistent with the conclusions drawn from the literature review conducted by Ronis and Walker (1989) in which the effects of 3MC-type inducers on avian species were reviewed and summarized.

6.1.2 NADH- and NADPH-Cytochrome *c* Reductase

6.1.2.1 Galliformes

The observed hepatic microsomal NADH- and NADPH-cytochrome *c* reductase activities were similar among the Galliformes species examined. The NADH- and NADPH-cytochrome *c* reductase activities measured in the chicken and quail hepatic microsomes were consistent with activities reported for these species by other researchers (Banton, 1990; Buynitzky, *et al.*, 1978; Buckpitt and Boyd, 1982; Pilch and Coomes, 1981; Rinsky and Perry, 1983; Carpenter, *et al.*, 1985; Riviere, 1983). No previously reported values for reductase activity could be found for the turkey. In the present study, no change in NADH- or NADPH-cytochrome *c* reductase activity was seen following treatment with βNF. Banton (1990) and Buckpitt and Boyd (1982) also found no change in NADPH-cytochrome *c* reductase activity in chicken and quail microsomes following pretreatment of the birds with βNF and 3MC, respectively. Banton (1990) also found no change in NADH-cytochrome *c* reductase activity in chicken hepatic microsomes associated with βNF administration. Buynitzky *et al.* (1978) also observed no change in NADPH cytochrome *c* reductase in the chicken following administration of 3MC. In contrast, Powis *et al.* (1976) demonstrated an increase in hepatic microsomal NADPH-cytochrome *c* reductase activity associated with 3MC administration in chicken embryos and chicks. Similarly, Carpenter and coworkers found a significant increase in NADPH-cytochrome *c* reductase activity in hepatic microsomes isolated from βNF-treated Japanese quail (Carpenter, *et al.*, 1985).

6.1.2.2 Anseriformes

In general, comparable levels of NADH- and NADPH-cytochrome *c* reductase activity were seen for the Anseriformes species studied. Differences noted between the Anseriformes species included a greater level of constitutive NADH-cytochrome *c* reductase activity in the goose compared to the Muscovy and a greater level of NADPH-cytochrome *c* reductase activity in the goose compared to the Muscovy duck following treatment with βNF. No change in NADH- or NADPH-cytochrome *c* reductase activity was noted in the Anseriformes species following βNF treatment. No studies were found in the literature in which the hepatic microsomal NADH- or NADPH-cytochrome *c* reductase activity was quantitated for the goose or the duck.

6.1.2.3 Comparison of Galliformes and Anseriformes

The hepatic microsomal NADPH-cytochrome *c* reductase activity levels observed in the present study were comparable for the Galliformes and Anseriformes species evaluated for both the control and the βNF treated birds. In contrast, a significantly ($p < 0.001$) greater ($\approx$ 2-fold) hepatic microsomal NADH-cytochrome *c* reductase activity was observed for the Anseriformes birds compared to the Galliformes birds.

6.1.2.4 Comparison of Rat and Avian Species

The NADH- and NADPH-cytochrome *c* reductase activity levels observed for the rat were consistent with levels reported in previous studies. As reported by other researchers, no change in hepatic microsomal NADH- or NADPH-cytochrome *c*

reductase activity was associated with βNF administration in the rat. This finding was also consistent with the observed response of the avian NADH- and NADPH-cytochrome *c* reductases. The NADH- and NADPH-reductase activity for both orders of birds were found to be comparable with that of the rat.

6.1.3 Overview

In summary, the constitutive components of the hepatic cytochrome P-450 monooxygenase system (with the exception of NADH-cytochrome *c* reductase) were demonstrated to be present at comparable levels in the representative Galliformes and Anseriformes species. Basal NADH-cytochrome *c* reductase activity was observed to be greater in the Anseriformes than in the Galliformes. Comparison of the individual species indicated that basal NADH-cytochrome *c* reductase activity was greater in the goose and Khaki Campbell duck compared to the chicken and turkey. In addition, following βNF, there was significantly greater NADH-cytochrome *c* reductase activity in the Khaki Campbell duck compared to the chicken and turkey. Other less significant differences between treatment groups included greater protein yield in the Khaki Campbell and Pekin ducks compared to the quail and greater P-450 content in the turkey compared to the Khaki Campbell duck and chicken.

In comparison of the avian and rat hepatic microsomal systems, there were some similarities and differences noted. Basal levels for microsomal protein yield were, in general, greater in the rat than in the avian species (chicken, quail, goose and

Muscovy duck). Constitutive cytochrome P-450 levels and NADH- and NADPH-cytochrome *c* reductase activities were comparable for the avian and mammalian classes. In response to βNF treatment, increases in microsomal protein yield were observed for the avian species but no increase in the amount of protein per gram of liver was seen for the rat. More dramatic increases in cytochrome P-450 levels were observed in the avian species compared to the rat. No change in NADH- or NADPH-cytochrome *c* reductase activity was observed for the birds or the rat. These findings are consistent with those of other studies. Microsomal protein level, cytochrome P-450 content, NADH-cytochrome *c* reductase activity and NADPH-cytochrome *c* reductase activity are presented for each species in Figures 6-1, 6-2, 6-3, and 6-4, respectively.

Upon review of the available studies characterizing the avian hepatic microsomal cytochrome P-450 monooxygenase system, it is readily apparent that there is considerably greater interexperimental variability in avian data than in data collected on laboratory species such as the rat. Interexperimental variability in data obtained on the constitutive avian hepatic microsomal system appears to be quantitative rather than qualitative. Comparison of studies characterizing the response of the avian hepatic microsomal system to treatment with βNF or 3MC, indicates that there are qualitative as well as quantitative differences present between the data sets. Qualitative differences in the response of the avian cytochrome P-450 system to 3MC-type inducers were noted for microsomal protein level (chicken and

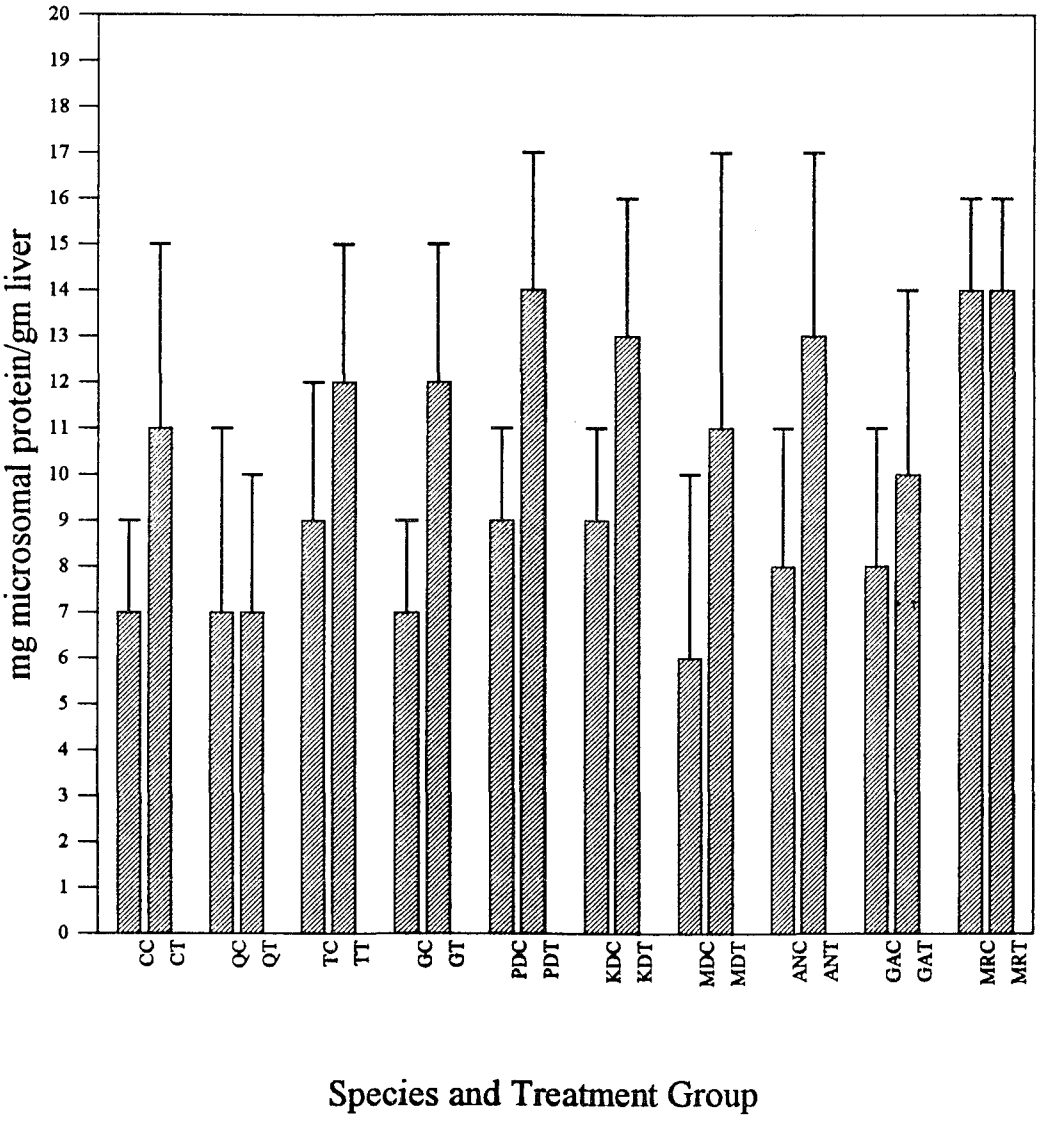

Figure 6-1 Hepatic microsomal protein content of control and BNF-treated avian species and male rats.

CC = Chicken Control; CT = Chicken Treated; QC = Quail Control; QT = Quail Treated; TC = Turkey Control; TT = Turkey Treated; GC = Goose Control; GT = Goose Treated; PDC = Pekin Duck Control; PDT = Pekin Duck Treated; KDC = Khaki Campbell Duck Control; KDT = Khaki Campbell Duck Treated; MDC = Muscovy Duck Control; MDT = Muscovi Duck Treated; ANC = Anseriformes Control; ANT = Anseriformes Treated; GAC = Galliformes Control; GAT = Galliformes Treated; MRC = Male Rat Control MRT = Male Rat Treated. These abbreviations are the same for Figures 6-2 through 6-16.

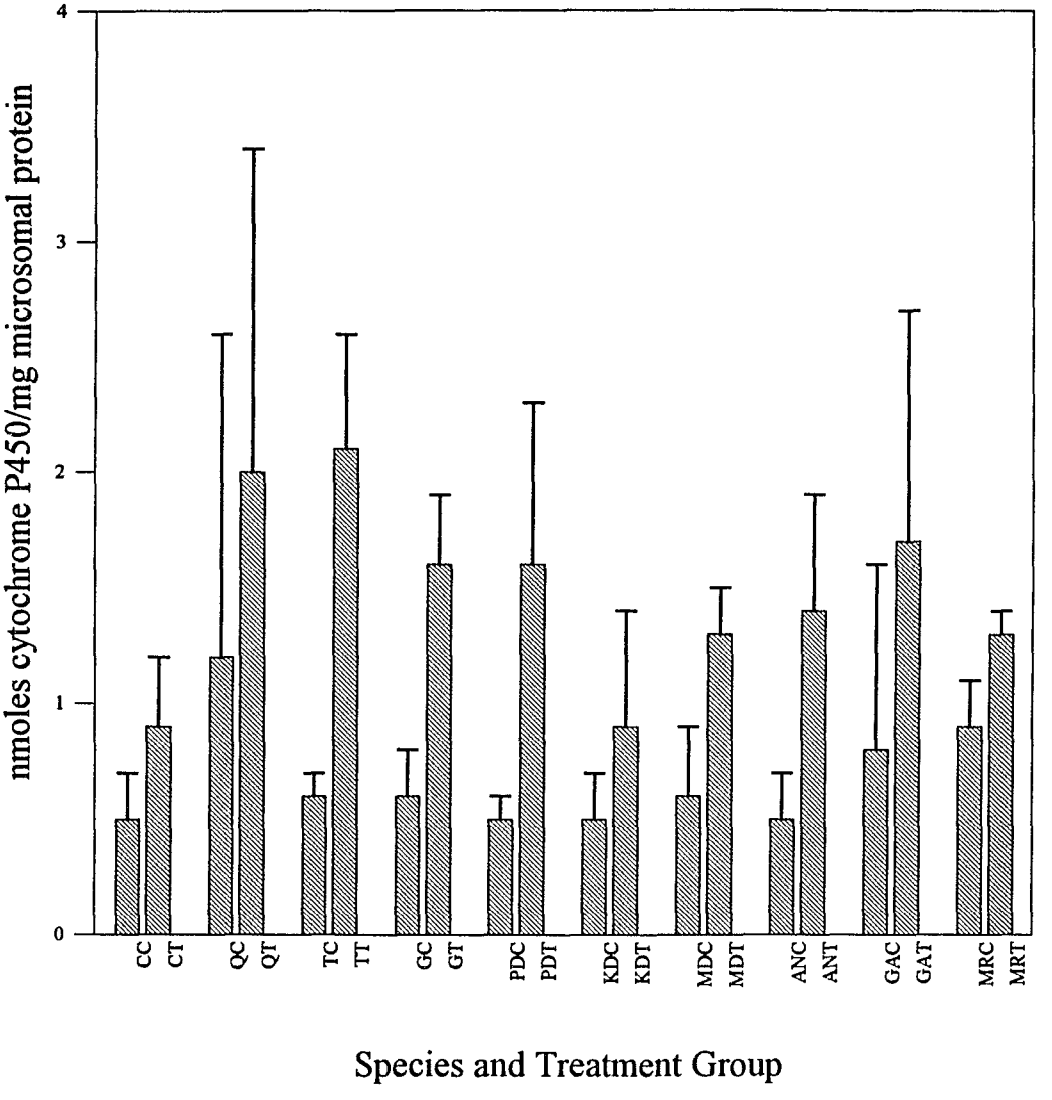

Figure 6-2 Hepatic microsomal cytochrome P-450 content of control and BNF-treated avian species and male rats. See Figure 6-1 for species identifications.

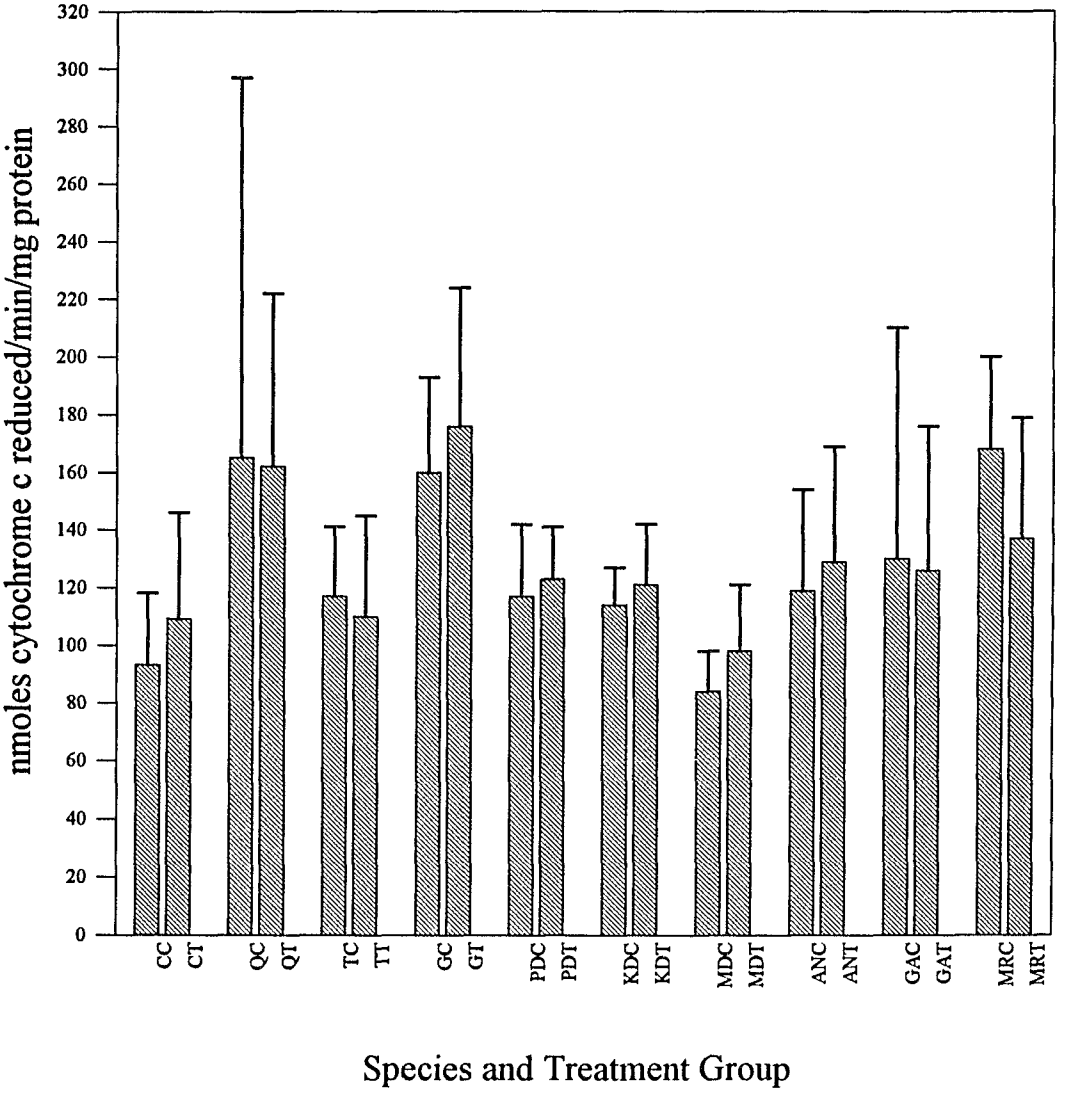

Figure 6-3 Rate of hepatic microsomal NADPH-cytochrome c reductase activity in control and BNF-treated avian species and male rats. See Figure 6-1 for species identifications.

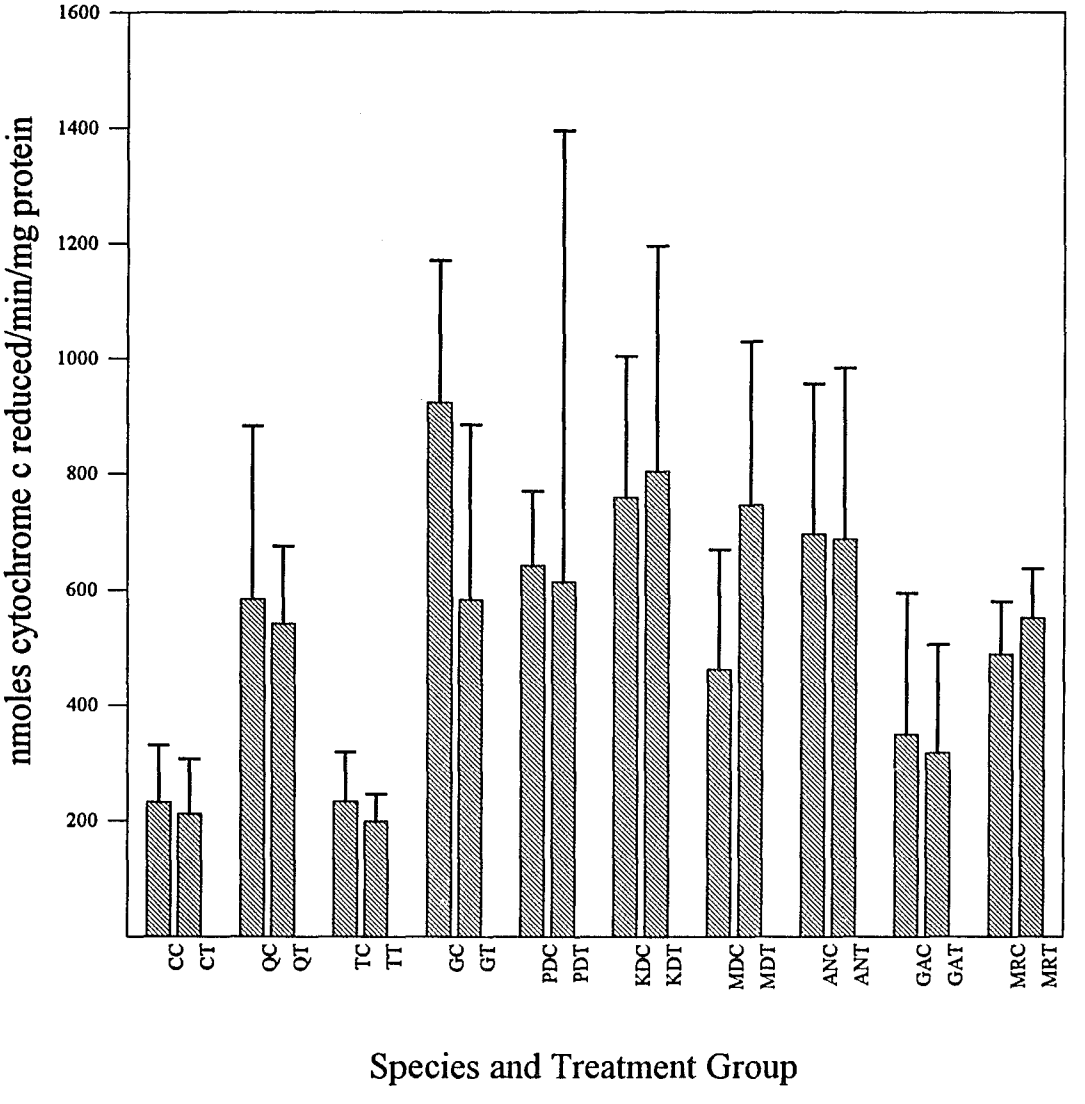

Figure 6-4 Rate of hepatic microsomal NADH-cytochrome c reductase activity in control and BNF-treated avian species and male rats. See Figure 6- 1 for species identifications.

quail), cytochrome P-450 content (quail), and NADPH-cytochrome *c* reductase activity (chicken and quail).

The interexperimental variability observed between studies presented in the literature may be attributable to greater genetic variability among birds; differences in the breed, sex or age of the birds studied; and differences in assay procedures or other experimental conditions such as the inducer used and/or the dosage administered and the degree of environmental control implemented (very limited data are available on the influences of temperature, light and seasonal variation on the cytochrome P-450 system of birds). Male and female birds of different breeds and of ages ranging from embryos of a few days of incubation to adults were used in the cytochrome P-450 studies present in the literature. A wide range of variability was also noted in the induction regimes employed by the various studies. Reported regimes for the administration of 3MC-type inducing agents included 50 mg/kg/day βNF for 3 days (Banton, 1990), 80 mg/kg βNF single dose (Haug, *et al.*, 1980), 80 mg/kg/day βNF for 2 days (Manning, *et al.*, 1990), 150 mg/kg/day βNF for 4 days (Carpenter, *et al.*, 1985), 20 mg/kg/day 3MC for 2 days (Buynitzky, *et al.*, 1978; Buckpitt and Boyd, 1982; Riviere, 1984), 15 mg/kg/day 3MC for 3 days (Neal, *et al.*, 1986), 20-80 mg/kg/day 3MC for 2 days (Buckpitt *et al.*, 1982) and 0.01-1000 μg/kg/day TCDD for 3 days (Althaus, *et al.*, 1972). While no studies have been conducted in avian species to characterize dose-response relationships for the induction of cytochrome P-450 components by βNF or other 3MC-type inducers, it has been demonstrated in the

rat that different dose regimes produce different hepatic cytochrome P-450 system responses. For example, maximal induction of EROD activity has been demonstrated to require a dose of 20 mg/kg βNF (Matthew and Houston, 1990) while maximal induction of AHH activity was observed to require a dose of 80 mg/kg βNF (Boobis, *et al.*, 1977). Similarly, Sawyer *et al.* (1986) found that the ED_{50} values for induction of AHH and EROD activities in the chicken by TCDD were 302 and 778 ng/kg, respectively. In addition, interspecies differences in the induction response have been observed. For example, Brunstrom and Lund (1988) found that the ED_{50} for the induction of AHH by 3,3'4,4'-tetrachlorobiphenyl was 10 times higher in the turkey than in the chicken. It was also noted during the literature review that there was considerable interexperimental variability in the time period allotted between treatment and microsomal isolation. Time periods for the various studies ranged from 24 hours to 10 days.

Observed interexperimental variability may also stem from the comparison of hepatic microsomal data obtained from birds treated with different inducers, *i.e.* βNF, 3MC, TCDD, 3,3',4,4'-tetrachlorobiphenyl, etc. Cytochrome P-450 studies conducted on the chicken suggest that the responses of the avian hepatic microsomal system to βNF and 3MC may not be identical. For example, Ehrich and Larsen (1983) observed a greater increase in the microsomal cytochrome P-450 level with 3MC treatment compared to βNF treatment whereas the reverse was noted for ANH activity. The same study also demonstrated that βNF treatment induced a broader spectrum of

hepatic cytochrome P-450 catalyzed activities than did 3MC. In the chicken embryo, Hamilton *et al.* (1983) and Althaus, *et al.* (1972) observed that the effective dose range for the induction of AHH activity by βNF was similar to that for induction by 3MC but that the maximal increase in activity produced by βNF was approximately half of that produced by 3MC. In addition, Ehrich *et al.* (1984) observed a difference in the time lapse required between treatment and the achievement of maximal induction of malaoxon detoxification for βNF (48 hours) and 3MC (96 hours).

6.2 Oxidative Activity of the Hepatic Cytochrome P-450 Monooxygenase System

6.2.1 Ethoxyresorufin *O*-Deethylase Activity

6.2.1.1 Galliformes

This study found that constitutive EROD activity was comparable among the Galliformes species studied. Basal microsomal EROD activity (based on specific and molar activities) measured in the chicken was consistent with the activities observed by Banton *et al.*(1989) and Banton (1990) but was lower (approximately 10-fold) than the activities (pmoles of resorufin formed/min/mg protein) reported by Sawyer *et al.* (1986), Riviere (1984), and Lee *et al.* (1986). The rate of metabolism of ethoxyresorufin (ER) (pmoles resorufin formed/min/mg protein) observed in this study for the Japanese quail was 4 to 8 times lower than the values reported by Carpenter *et al.* (1985), Riviere *et al.* (1983) and Riviere *et al.* (1985). No previously published values for the hepatic microsomal metabolism of ER could be found for the turkey.

Following βNF treatment, EROD activity seen for the turkey was significantly greater than that demonstrated by the Japanese quail. The greatest induction of EROD metabolism among the Galliformes species was observed in the chicken (39-fold based on specific activity). This increase in activity is consistent with the findings of Sawyer *et al.* (1986) who reported a 37.5-fold increase in EROD activity in the chicken following treatment with TCDD. Banton (1990) demonstrated a slightly lower increase (27.5-fold) in hepatic EROD activity associated with βNF treatment while Rifkind *et al.* (1984) reported a slightly higher increase (50-fold) following treatment with 3,3',4,4'-tetrachlorobiphenyl. In contrast, Quilley and Rifkind (1986) demonstrated a dramatic increase in EROD activity in the chicken of 200-fold with TCDD administration while Brunstrom (1986) only observed a 2.5-fold increase following treatment with 3,3',4,4'-tetrachlorobiphenyl. Based on turnover number, a 21.4-fold increase in the rate of ER deethylation was observed in the chicken which is considerably higher than the 7.1-fold increase reported by Banton (1990).

In the present study, the Japanese quail demonstrated a 16-fold increase in ER metabolism (based on specific activity) following treatment with βNF. Similarly, Carpenter, *et al.* (1985) reported a 13-fold increase in the rate of ER metabolism in this species following treatment with βNF. Riviere (1984) observed an increase in EROD activity of only 2.8-fold and 2.5-fold following administration of βNF and 3MC, respectively. In contrast to these observations, Neal *et al.* (1986) reported a 53 percent decease in EROD activity in the Japanese quail following treatment with

3MC. No reports on the effects of 3MC-type inducers on hepatic microsomal ER metabolism were found in the literature for the turkey.

6.2.1.2 Anseriformes

In the present study, basal hepatic microsomal ER metabolism (based on specific and molar activities) was found to be similar among the Anseriformes species studied. Upon review of the literature, no reported values for constitutive EROD activity were located for any of the Anseriformes species examined in this study.

All of the Anseriformes species demonstrated induction of hepatic microsomal EROD activity following treatment with βNF. Comparable increases in deethylase activity were observed for the goose, Pekin duck and Khaki Campbell duck while the increase in activity observed for the Muscovy duck was approximately half of that seen for the other species. No reported values for hepatic ER metabolism were discovered in the literature for the goose, Pekin duck, Khaki Campbell duck, or Muscovy duck. Brouwer (1991) reported a 57-fold increase in EROD activity (based on specific activity) in the Eider duck following exposure to 3,3',4,4'-tetrachlorobiphenyl. This reported increase is considerably higher that the increases observed here with βNF for the Pekin (20-fold), Khaki Campbell (16-fold) and Muscovy (9-fold) ducks.

6.2.1.3 Comparison of Galliformes and Anseriformes

The constitutive rate of ER metabolism demonstrated by the Anseriformes order was approximately twice that seen for the Galliformes order. In contrast, induced EROD activities were similar for the two orders. However, when the treatment groups of each species were evaluated individually, some significant differences were observed between the minor species of these orders (Pekin duck > quail). Both avian orders demonstrated an induction of EROD activity following treatment with βNF but the level of induction was approximately twice as great in the Galliformes as in the Anseriformes.

6.2.1.4 Comparison of Rat and Avian Species

Results of this study indicated that the constitutive EROD activities of the avian species were comparable to that of the rat. However, following βNF treatment, the level of hepatic microsomal EROD activity demonstrated by the rat was significantly greater (≈3-fold) than that seen for the avian species. In addition, the degree of induction observed in the rat was 4 times greater than that shown by the avian species based on specific activity and 6 times greater based on turnover number. The increase in EROD activity observed in the present study for the rat is consistent with those seen by other researchers (Birgersson, *et al.*, 1985; Burke *et al.*, 1985; Burke and Mayer, 1974; Burke and Mayer 1983; Guengerich, *et al.*, 1982; Guengerich, *et al.*, 1982a; Ryan, *et al.*, 1982).

Ethoxyresorufin *O*-deethylase activity for each species and order is presented in Figures 6-5.

6.2.2 Benzo[*a*]pyrene Hydroxylase Activity and Metabolic Profile

6.2.2.1 Galliformes

Although similar patterns of B[a]P metabolism were observed for the Galliformes, quantitative differences in metabolite formation were noted between the species. In general, constitutive benzo[*a*]pyrene hydroxylase activity (based on total metabolite production) was observed to be significantly greater in the turkey than the chicken and quail. Specifically, significantly greater quantities of phenols (3-OH B[*a*]P and 9-OH B[*a*]P) were produced by the turkey hepatic microsomes than by microsomes isolated from the chicken and quail. The present study demonstrated that basal AHH activity in the turkey was 5.7-fold (based on specific activity) and 4.4-fold (based on turnover number) greater than activity levels observed for the chicken. Consistent with this finding, Brunstrom and Lund (1988) found that constitutive AHH activity in the turkey was 3.5 times greater than that of the chicken. It is also interesting to note that these researchers observed that the turkey required a greater dose of 3,3',4,4'-tetrachlorobiphenyl to achieve maximal induction of AHH activity than did the chicken.

Induction of B[*a*]P metabolism was observed in the chicken and quail but not in the turkey. The greatest increase in total metabolite production was seen in the chicken

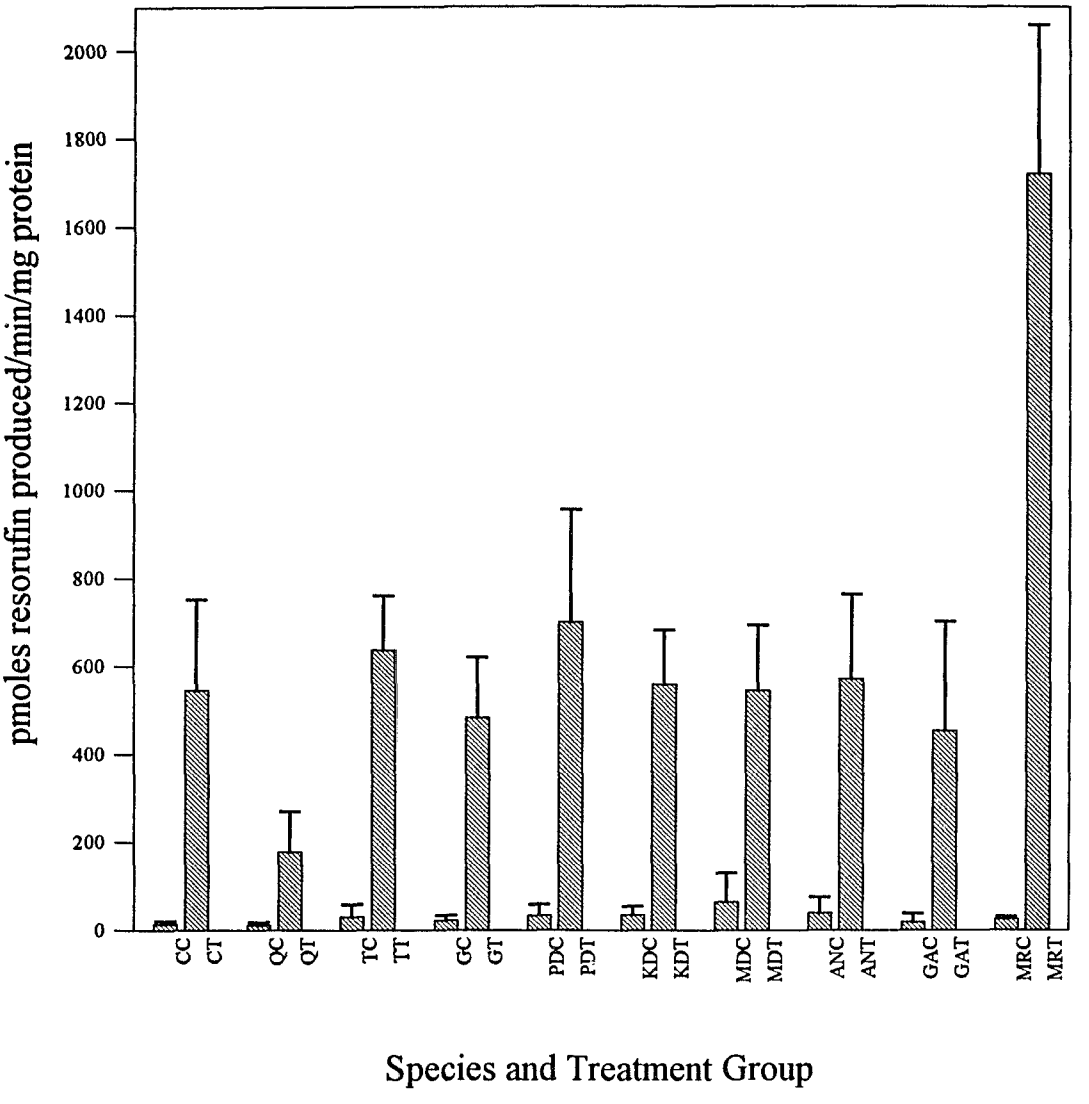

Figure 6-5 Rate of hepatic microsomal ethoxyresorufin O-deethylase activity in control and BNF-treated avian species and male rats. See Figure 6-1 for species identifications.

(5-fold based on specific activity, 2.5-fold based on turnover number) followed by the quail (2.3-fold based on specific activity, 1.7-fold based on turnover number). Other researchers have reported increases in B[*a*]P hydroxylase activity following treatment of the chicken with 3MC-type inducers (3MC, TCDD, B[*a*]P, βNF, and 3,3',4,4'-tetrachlorobiphenyl). The reported increases ranged from 2 to 40-fold over control values (Buynitzky, *et al.*, 1978; Brunstrom, *et al.*, 1986; Dogra and Israels, 1987; Haug, *et al.*, 1980; Poland and Glover, 1977; Althaus, *et al.*, 1972; Hamilton, *et al.*, 1983; Ehrich and Larsen, 1983; Rosanoff, *et al.*, 1984; Strittmatter and Umberger, 1969; Powis, *et al.*, 1976; Sawyer, *et al.*, 1986; Darbey, *et al.*, 1984; Darbey, *et al.*, 1985; Darbey, *et al.*, 1986; Rifkind, *et al.*, 1984; Topp and van Bladeren, 1986). Only one study was found in the literature in which AHH activity was examined in the Japanese quail. Carpenter, *et al.* (1985) demonstrated a 13-fold increase in AHH activity over the basal level following βNF administration. This reported increase is greater than the increase of 2.3-fold observed in the present study. In contrast to the chicken and quail, no change in total metabolite formation was seen for the turkey in terms of specific activity and based on turnover number, a significant decrease in metabolite production was observed. No studies were located in the literature which quantitated the increase in AHH activity in the turkey associated with the administration of 3MC-type inducers.

Following βNF treatment, the metabolic profiles generated by the Galliformes species were qualitatively similar to each other and to the control profiles (with the addition

of 9,10-diol formation). Specific quantitative changes observed in the metabolic profiles of the chicken and quail included significant increases in the production of 3-OH B[*a*]P, 9-OH B[*a*]P and B[*a*]P 7,8-diol. The only qualitative change noted was the formation of the 9,10-diol metabolite. For the turkey, changes in product distribution included a significant increase in B[*a*]P 7,8-diol, a significant decrease in 3-OH B[*a*]P, no change in 9-OH, and the formation of 9,10-diol. Shifts in the metabolic profiles due to changes in the relative production of individual metabolites were noted following βNF treatment. In the chicken and quail, there was a relative increase in the production of dihydrodiols and a relative decrease in the production of phenols. For the turkey, the shift in product distribution included a relative increase in the formation of dihydrodiols and 9-OH B[*a*]P and a relative decrease in 3-OH B[*a*]P production. Topp and van Bladeren (1986) examined the metabolism of B[*a*]P by primary cultures of control and βNF-treated chick embryo hepatocytes. The product distributions observed by these researchers were similar, but not identical, to those of this study. For the control hepatocytes, metabolite formation, in order of decreasing magnitude, included 3-OH > 9-OH > 9,10-diol > 7,8-diol. For control chicken microsomes, this study found metabolite production of the following sequence: 3-OH > 9-OH > 7,8-diol with no 9,10-diol formation. Following βNF administration, a metabolic profile of 3-OH ≈ 9-OH > 7,8-diol > 9,10-diol was observed by Topp and van Bladeren while a product distribution of 3-OH > 9-OH > 7,8-diol > 9,10-diol was seen in the present study.

The results of this study illustrate a significant quantitative difference in the *in vitro* metabolism of B[*a*]P between the two major food-producing Galliformes species (chicken and turkey). In addition, the findings of this study suggest that the characteristics of B[*a*]P metabolism in control and βNF-treated quail were qualitatively and quantitatively more similar to those of the chicken than to those of the turkey. Metabolite formation by the control and βNF-treated Galliformes species is shown in Figures 6-6 through 6-8.

6.2.2.2 Anseriformes

Overall, basal AHH activity, based on total B[*a*]P metabolite formation, was comparable among the Anseriformes species studied. However, quantitative as well as qualitative differences in individual metabolite formation were noted between the Anseriformes birds. In general, the constitutive metabolic profiles were similar for the duck species examined. The most notable difference observed among these birds was the constitutive formation of the B[*a*]P 9,10-diol metabolite by the Khaki Campbell duck (the Khaki Campbell was the only avian species which produced this metabolite). Another notable difference in the metabolism of B[*a*]P among the Anseriformes was found in the goose. This species demonstrated a basal metabolic profile that did not include the formation of either of the dihydrodiol metabolites examined.

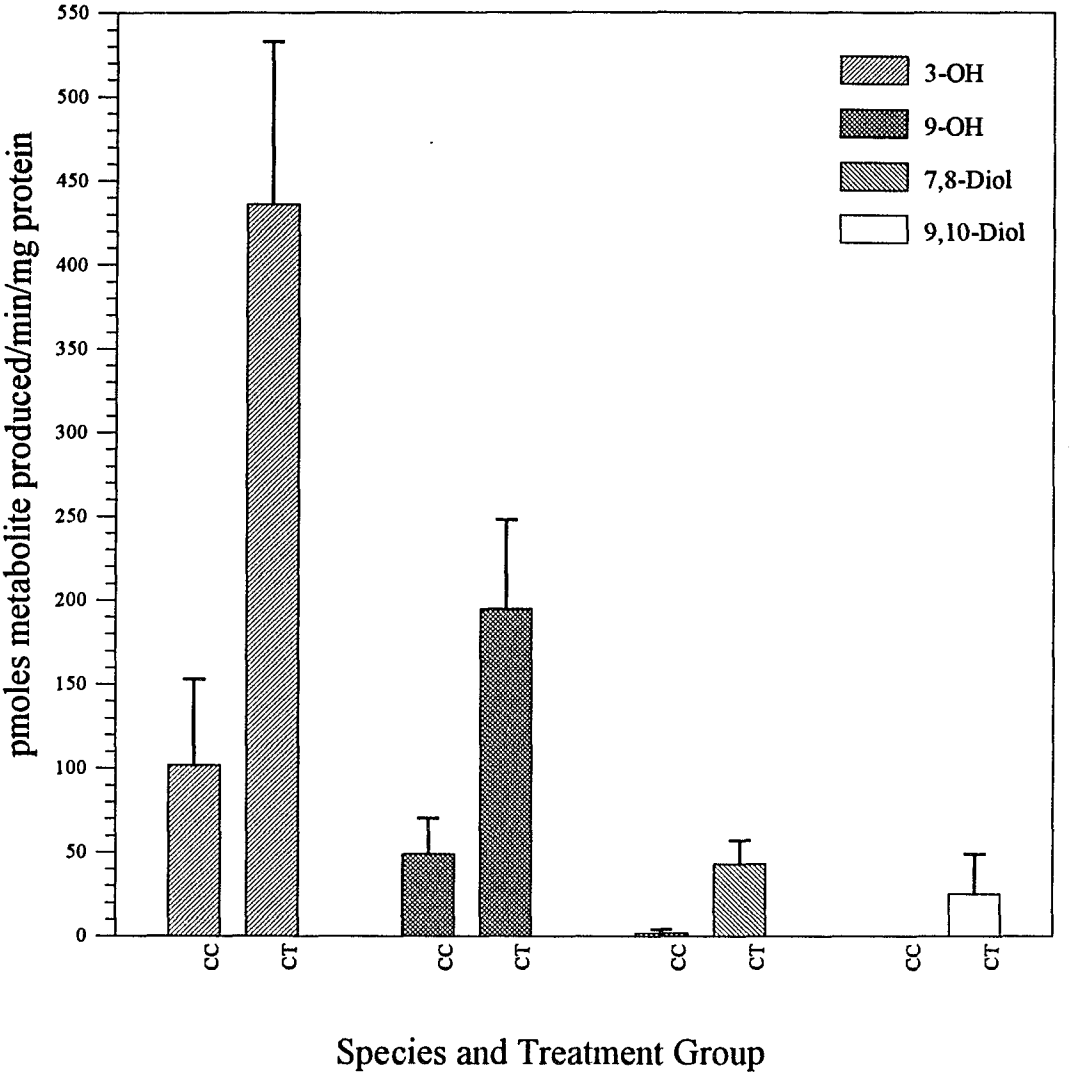

Figure 6-6 Hepatic microsomal production of benzo[a]pyrene metabolites in control (CC) and BNF-treated (CT) chickens.

3-OH = 3-hydroxybenzo[a]pyrene; 9-OH = 9-hydroxybenzo[a]pyrene
7,8-Diol = benzo[a]pyrene 7,8-dihydrodiol; 9,10-Diol = benzo[a]pyrene 9,10-dihydrodiol
These abbreviations are the same for Figures 6-6 through 6-15.

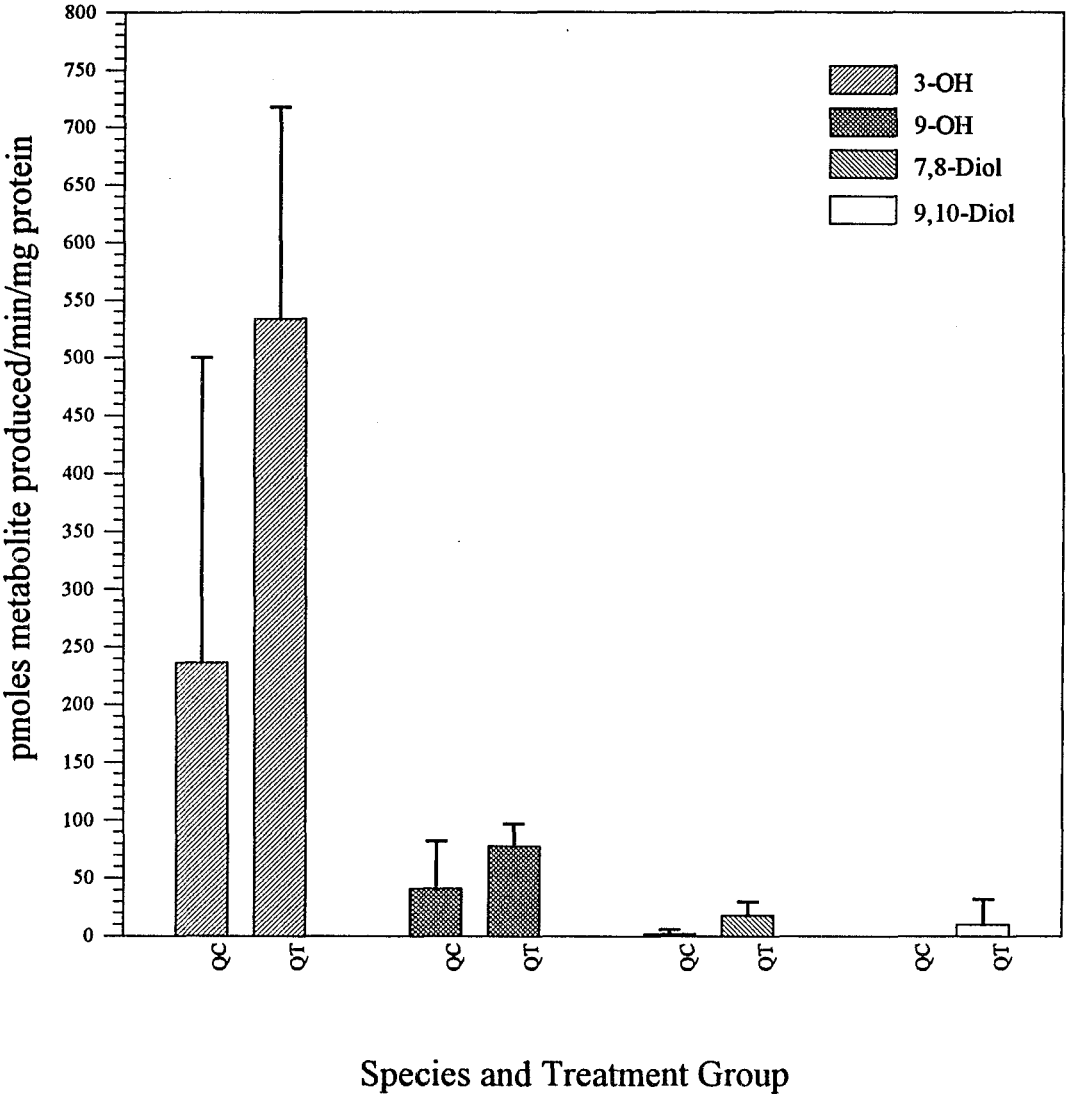

Figure 6-7 Hepatic microsomal production of benzo[a]pyrene metabolites in control (QC) and BNF-treated (QT) Japanese quail. See Figure 6-6 for metabolite identifications.

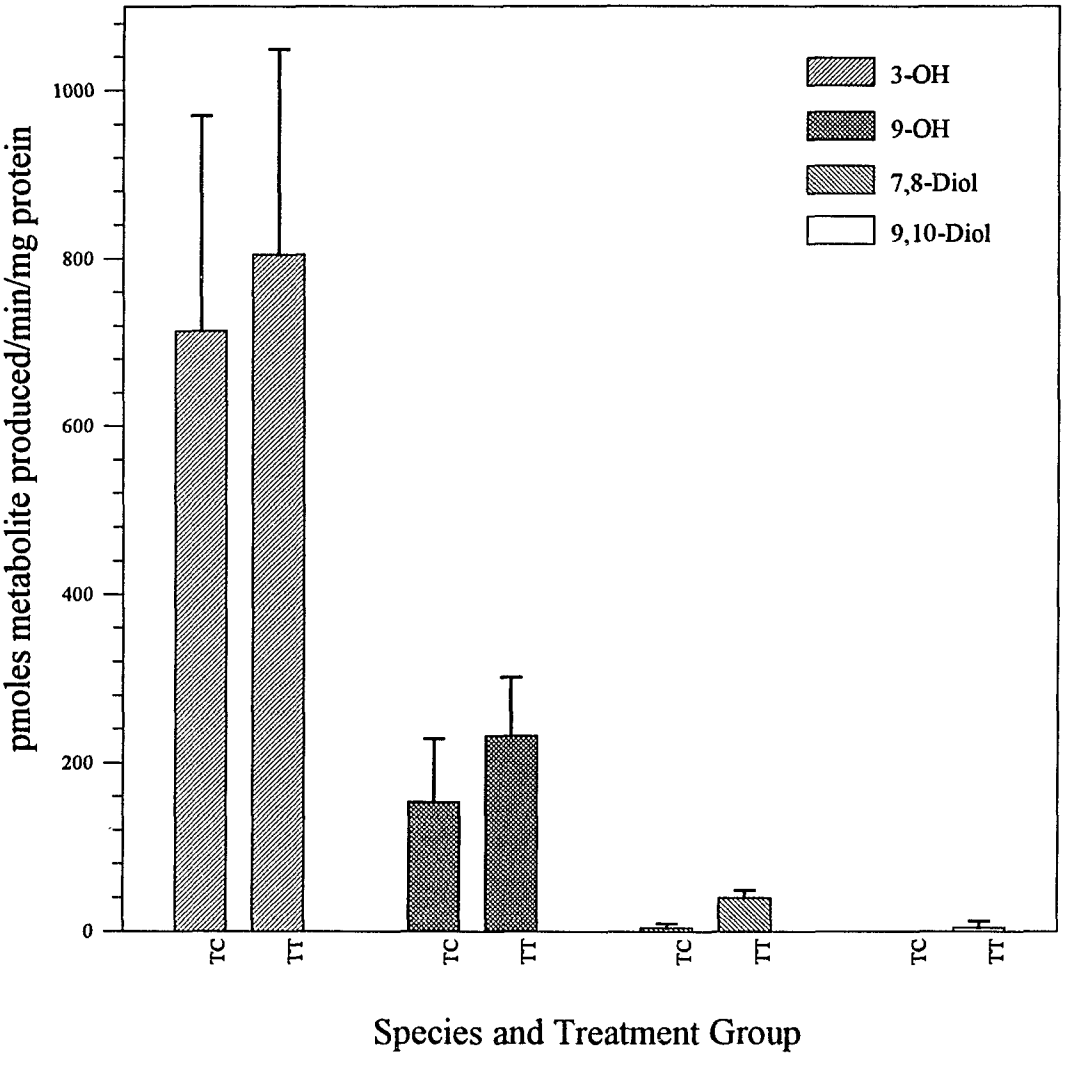

Figure 6-8 Hepatic microsomal production of benzo[a]pyrene metabolites in control (TC) and BNF-treated (TT) turkeys. See Figure 6-6 for metabolite identifications.

In response to βNF administration, induction of AHH activity (pmoles of total metabolites produced/min/mg protein) was seen in only one Anseriformes species, the Pekin duck. A decrease in metabolite production was observed in the goose based on turnover number; however, no change in production was seen based on specific activity. For the Khaki Campbell and Muscovy ducks, no differences in activity were noted respective to control values. Profiles of metabolite formation following βNF were similar among the duck species examined. The only differences noted were the quantities of individual metabolites produced. In contrast, the metabolic profile of the βNF-treated goose was dissimilar to those observed for the ducks and for the control goose. Relative changes observed in metabolite formation for the Anseriformes included a relative increase in dihydrodiol and 9-OH B[*a*]P and a relative decrease in 3-OH B[*a*]P. There were no studies found during the literature review which examined AHH activity in the Anseriformes species represented in this study. Metabolite formation by the control and βNF-treated Anseriformes species is illustrated in Figures 6-9 through 6-12.

6.2.2.3 Comparison of Galliformes and Anseriformes

Basal B[*a*]P hydroxylase activities were comparable for the Anseriformes and Galliformes birds. In addition, categorization and comparison of the data based on order indicated that the constitutive profiles of B[*a*]P metabolism for the Anseriformes and Galliformes (with the exception of basal B[*a*]P 9,10-diol formation by the Khaki Campbell duck and the lack of 7,8-diol formation by the goose) were similar.

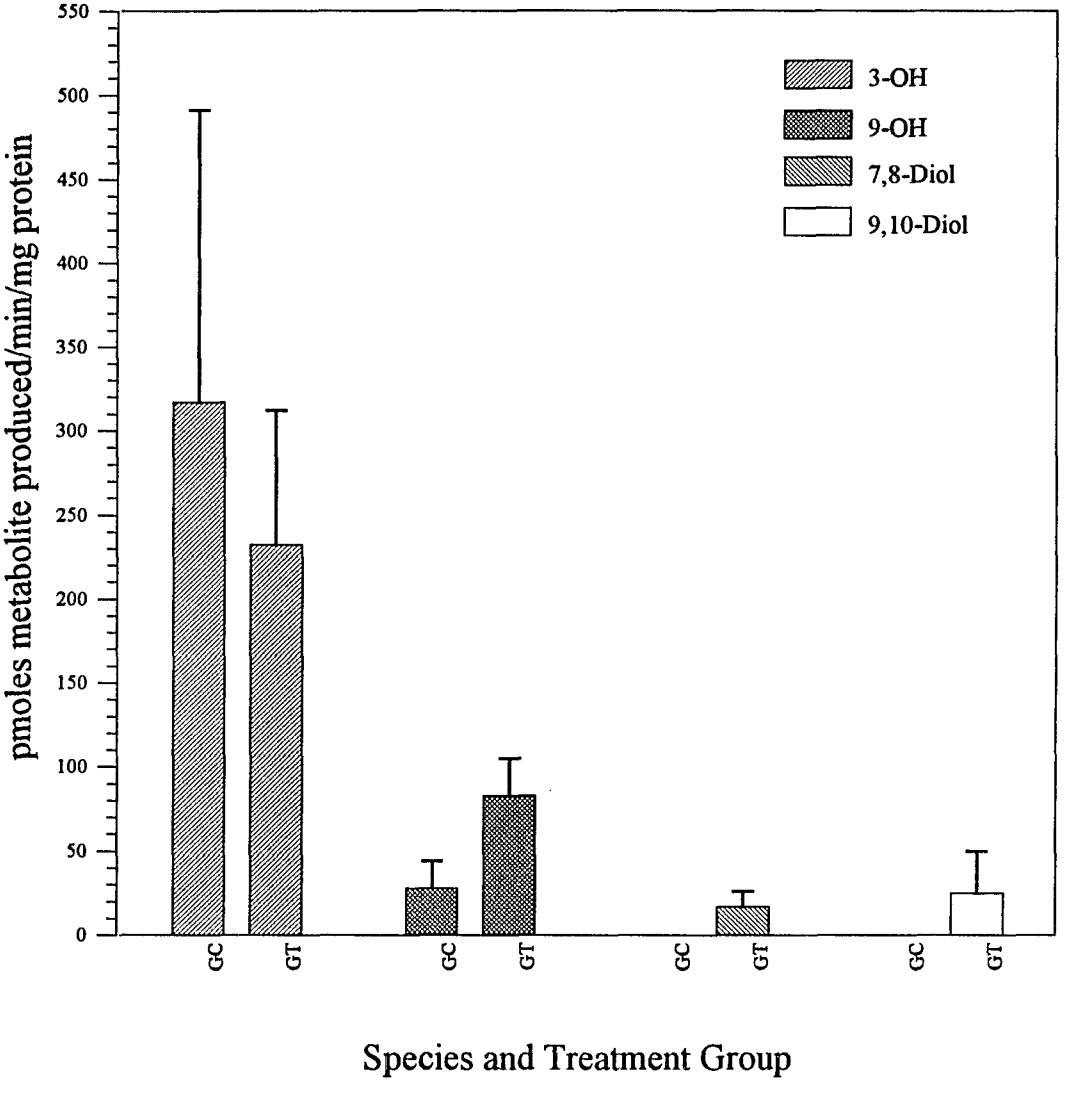

Figure 6-9 Hepatic microsomal production of benzo[a]pyrene metabolites in control (GC) and BNF-treated (GT) geese. See Figure 6-6 for metabolite identifications.

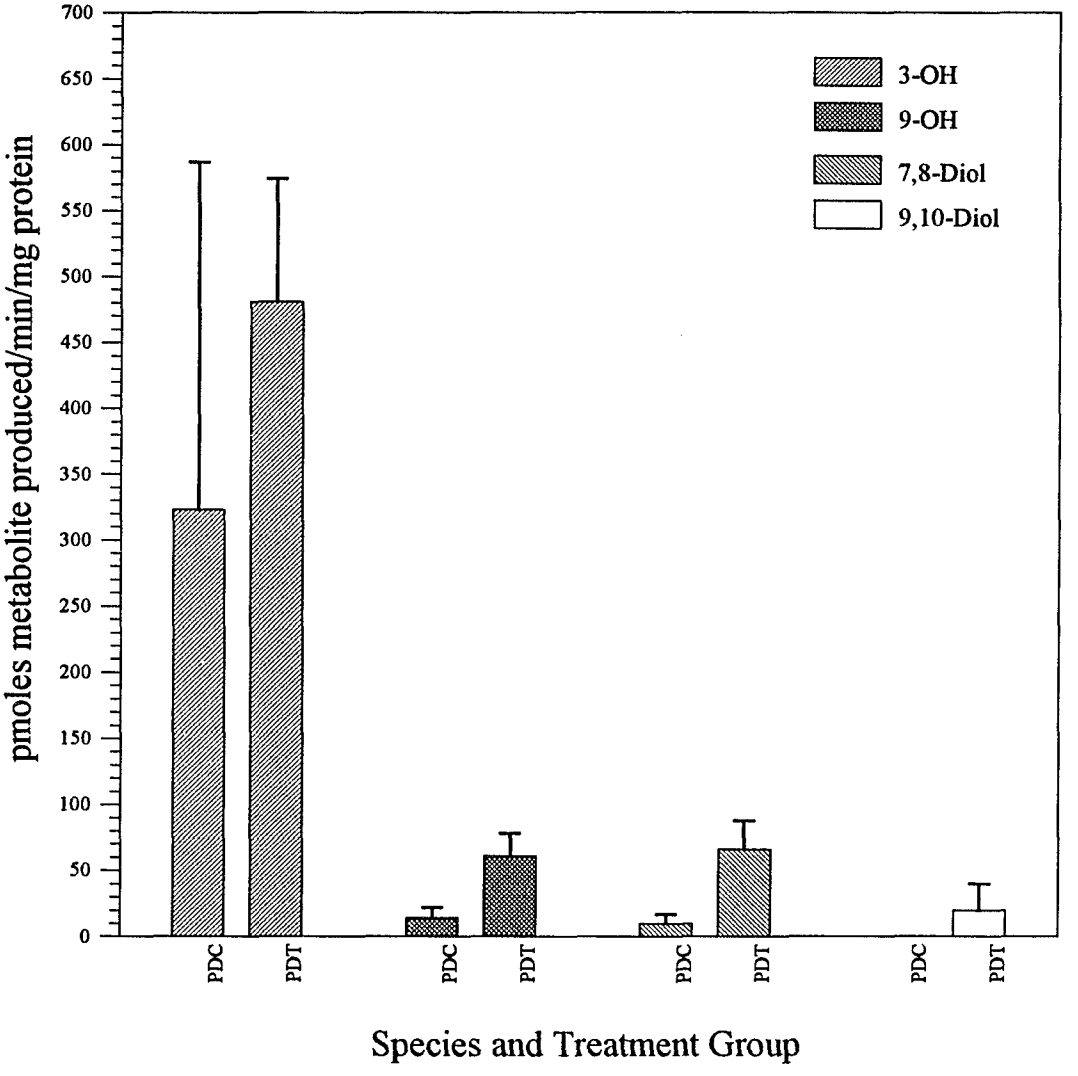

Figure 6-10 Hepatic microsomal production of benzo[a]pyrene metabolites in control (PDC) and BNF-treated (PDT) Pekin ducks. See Figure 6-6 for metabolite identifications.

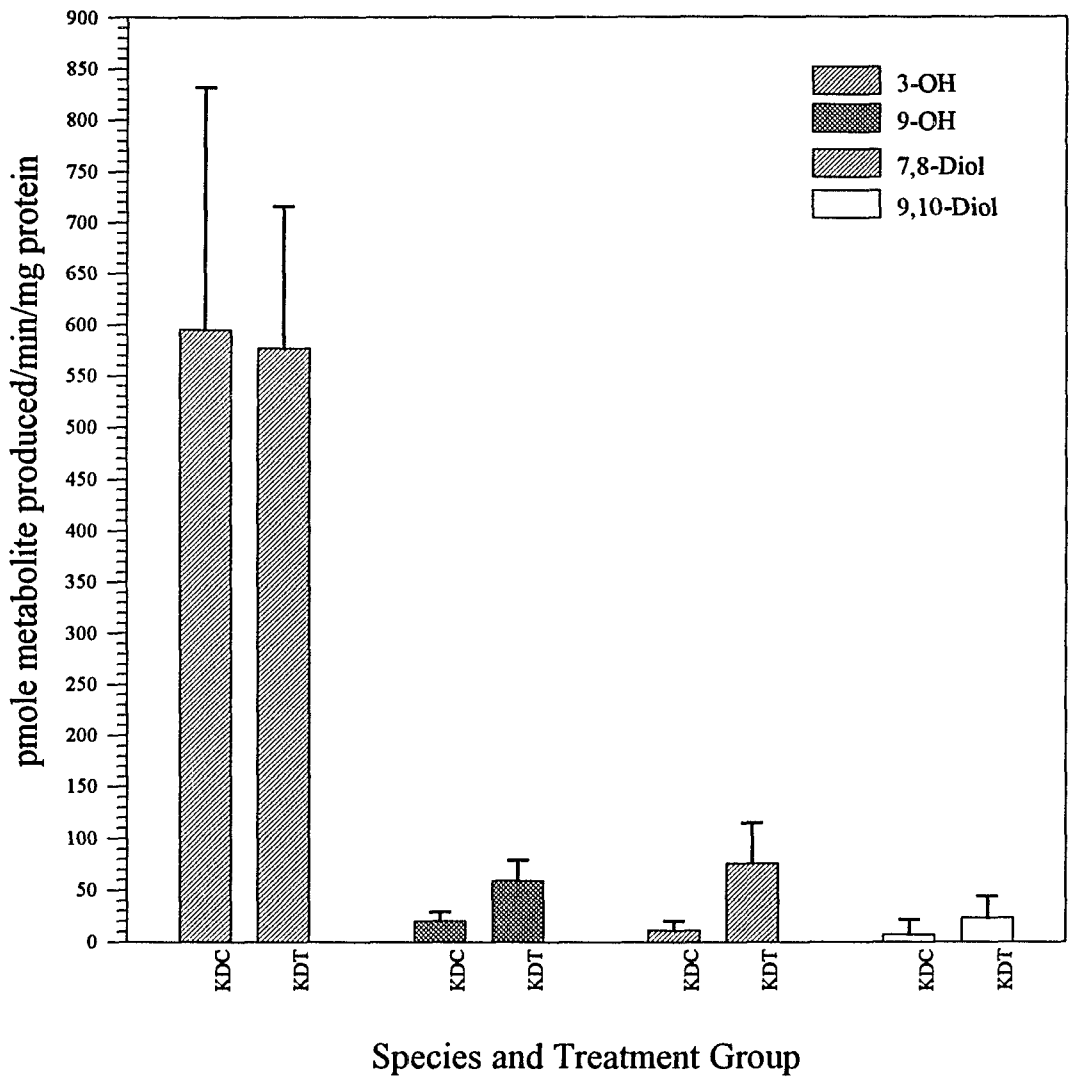

Figure 6-11 Hepatic microsomal production of benzo[a]pyrene metabolites in control (KDC) and BNF-treated (KDT) Khaki Campbell Ducks. See Figure 6-6 for metabolite identifications.

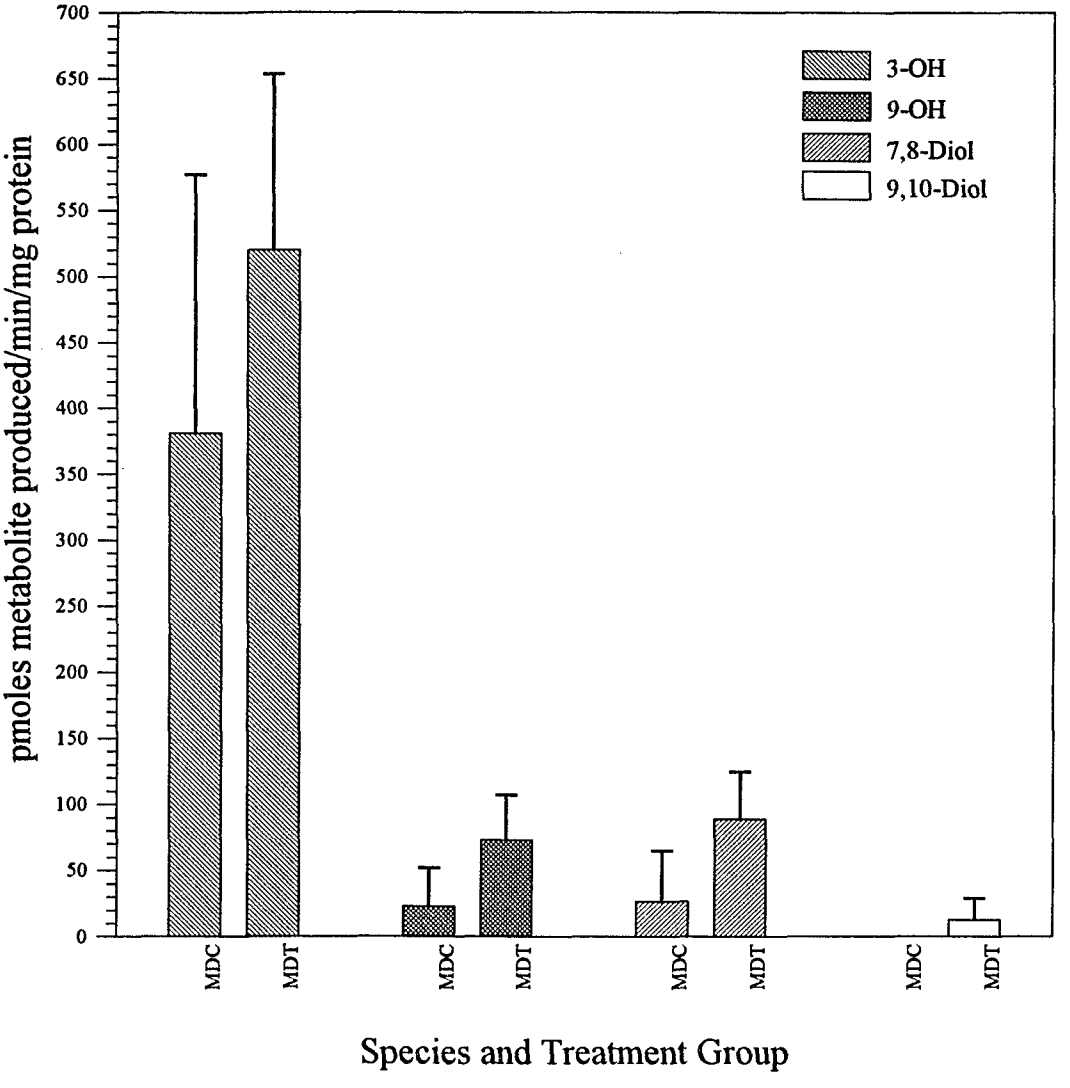

Figure 6-12 Hepatic microsomal production of benzo[a]pyrene metabolites in control (MDC) and BNF-treated (MDT) Muscovy ducks. See Figure 6-6 for metabolite identifications.

Following treatment with βNF, induction of AHH activity (based on specific activity) was demonstrated for both orders. However, based on turnover number, a decrease in total metabolite production was noted for the Anseriformes. Benzo[*a*]pyrene hydroxylase activity following βNF was significantly greater in the Galliformes than in the Anseriformes. The observed shift in metabolite formation was consistent between the avian orders (increased production of dihydrodiols and 9-OH B[*a*]P and decreased production of 3-OH B[*a*]P). Metabolite formation by the Galliformes and Anseriformes orders is illustrated in Figures 6-13 and 6-14, respectively.

Quantitative and qualitative differences in B[*a*]P metabolism were noted between major and minor species of the Galliformes and Anseriformes orders and also between the minor species of these orders. Interspecies comparisons between these orders indicated that product distributions of B[*a*]P metabolism by control microsomes were similar for the chicken, turkey, Japanese quail, Pekin duck, and Muscovy duck (3-OH > 9-OH > 7,8-diol). Qualitative differences in metabolite formation were noted for the goose (3-OH > 9-OH) and the Khaki Campbell duck (3-OH > 9-OH > 7,8-diol ≈ 9,10-diol). In βNF-treated birds, the patterns of B[*a*]P metabolism were similar for the Galliformes species (3-OH > 7,8-diol > 9-OH > 9,10 diol) and for the Anseriformes duck species (3-OH > 7,8-diol ≈ 9-OH > 9,10-diol). The metabolic profiles produced by the control (3-OH > 9-OH) and treated (3-OH > 9-OH > 9,10-diol > 7,8-diol) goose were unlike any profiles produced by the other avian species.

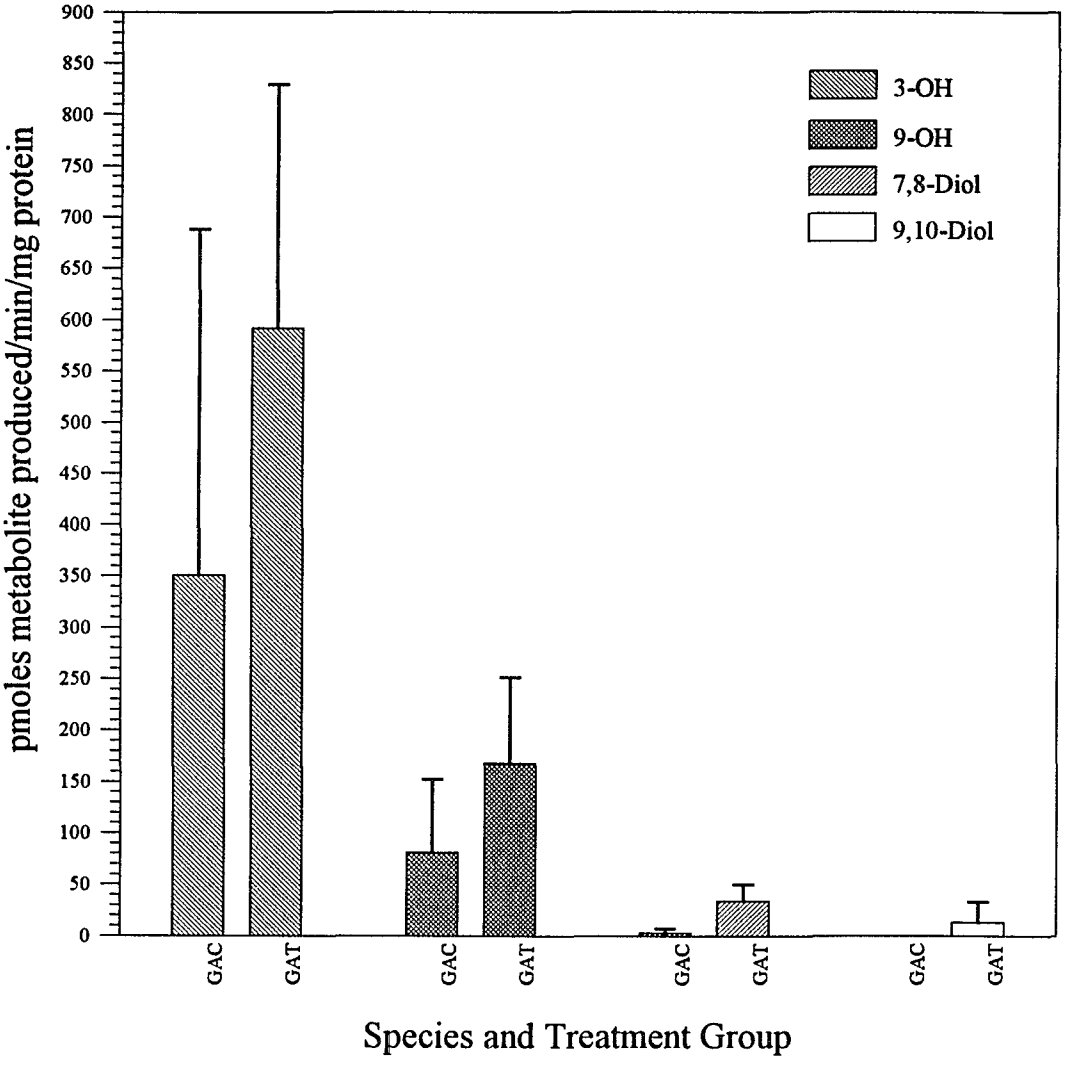

Figure 6-13 Hepatic microsomal production of benzo[a]pyrene metabolites in control (GAC) and BNF-treated (GAT) Galliformes species. See Figure 6-6 for metabolite identifications.

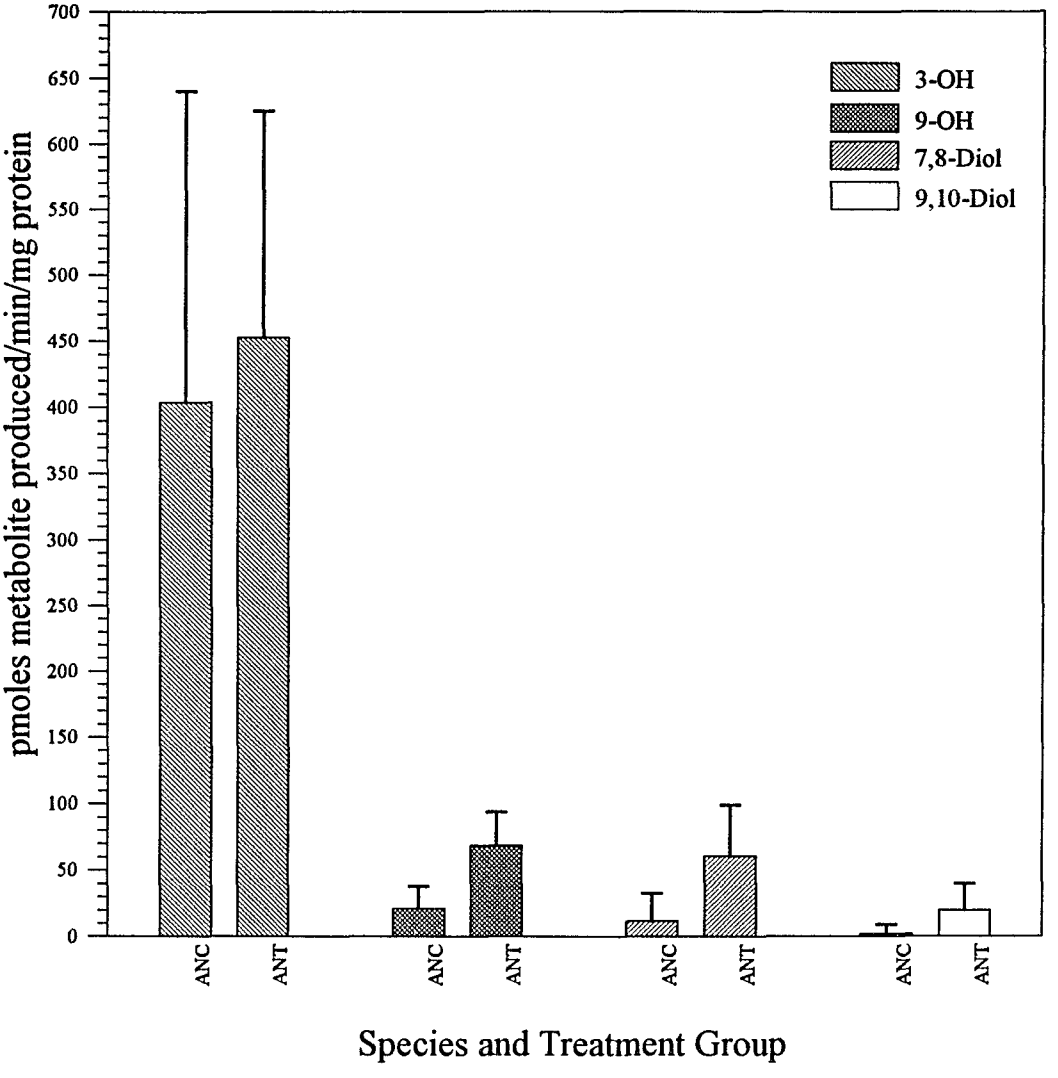

Figure 6-14 Hepatic microsomal production of benzo[a]pyrene metabolites in control (ANC) and BNF-treated (ANT) Anseriformes species. See Figure 6-6 for metabolite identifications.

6.2.2.4 Comparison of Rat and Avian Species

Constitutive benzo[*a*]pyrene hydroxylase activities observed for the avian species were comparable to that of the rat with the exception of the turkey and Khaki Campbell duck which exhibited greater activities (pmole of metabolites/min/nmole P-450). In response to treatment with βNF, a 1.7-fold increase in total metabolite formation (based on specific activity) was observed in the rat. This observation falls within the lower end of the range of values reported in the literature for increases (1.6 to 14.4-fold over controls) in AHH activity associated with the administration of 3MC-type inducers (Birgersson, *et al.*, 1985; Poland and Glover, 1977; Guengerich, *et al.*, 1982; Keller, *et al.*, 1987; Winston, *et al.*, 1991; Wroblewski, *et al.*, 1988). In comparison to the rat, greater increases in total metabolite formation (pmoles of metabolites/min/mg of protein) following βNF were observed in the chicken (5-fold) and Japanese quail (2.3-fold). When evaluated on the basis of turnover number, no change in benzo[*a*]pyrene hydroxylase activity was seen in the rat. In contrast, increases in turnover number were observed for the hepatic microsomes of the chicken (2.5-fold) and quail (1.7-fold) and decreases were seen for the turkey and goose microsomes.

For this study, the production of B[*a*]P metabolites by control and βNF-treated rats included, in order of decreasing magnitude, 3-OH > 9,10-diol > 9-OH > 7,8-diol. Qualitative as well as quantitative differences were noted between the profiles of B[a]P metabolism published in the literature. For the purpose of comparison, the following

review of the metabolic profiles presented in the literature focuses only on the four metabolites that were quantitated in the present study. The profile demonstrated by Wroblewski, *et al.* (1988) for basal B[*a*]P metabolism in the rat was consistent with the findings of this study. However, these researchers observed a shift in metabolite production (9,10-diol > 7,8-diol ≈ 3-OH > 9-OH) following TCDD administration which was not observed in the study presented here. Similar, but not identical, profiles (3-OH > 9,10-diol > 7,8-diol > 9-OH) were seen for control and treated (3MC-type inducing agents) rats by Wong, *et al.* (1986), Winston, *et al.*, (1991), and Sagami, *et al.*, (1987). In contrast, the metabolic profiles reported by Cavaliere and coworkers (1988) were dissimilar to the findings of this study. For control rats, the profile included 3-OH > 9-OH > 7,8-diol ≈ 9,10-diol and following TCDD treatment, 9,10-diol > 7,8-diol ≈ 3-OH > 9-OH (note - this profile is similar to the profile observed by Wroblewski presented above). Metabolite formation by the control and βNF-treated rat is illustrated in Figure 6-15. Total metabolite formation for all the species studied is presented in Figure 6-16.

6.2.3 Overview

The results of this study indicate, that for the representative Galliformes and Anseriformes species, the avian hepatic cytochrome P-450 monooxygenase system has the capacity to perform *O*-deethylation of ethoxyresorufin and hydroxylation of benzo[*a*]pyrene and that changes in these cytochrome P-450-dependent activites are

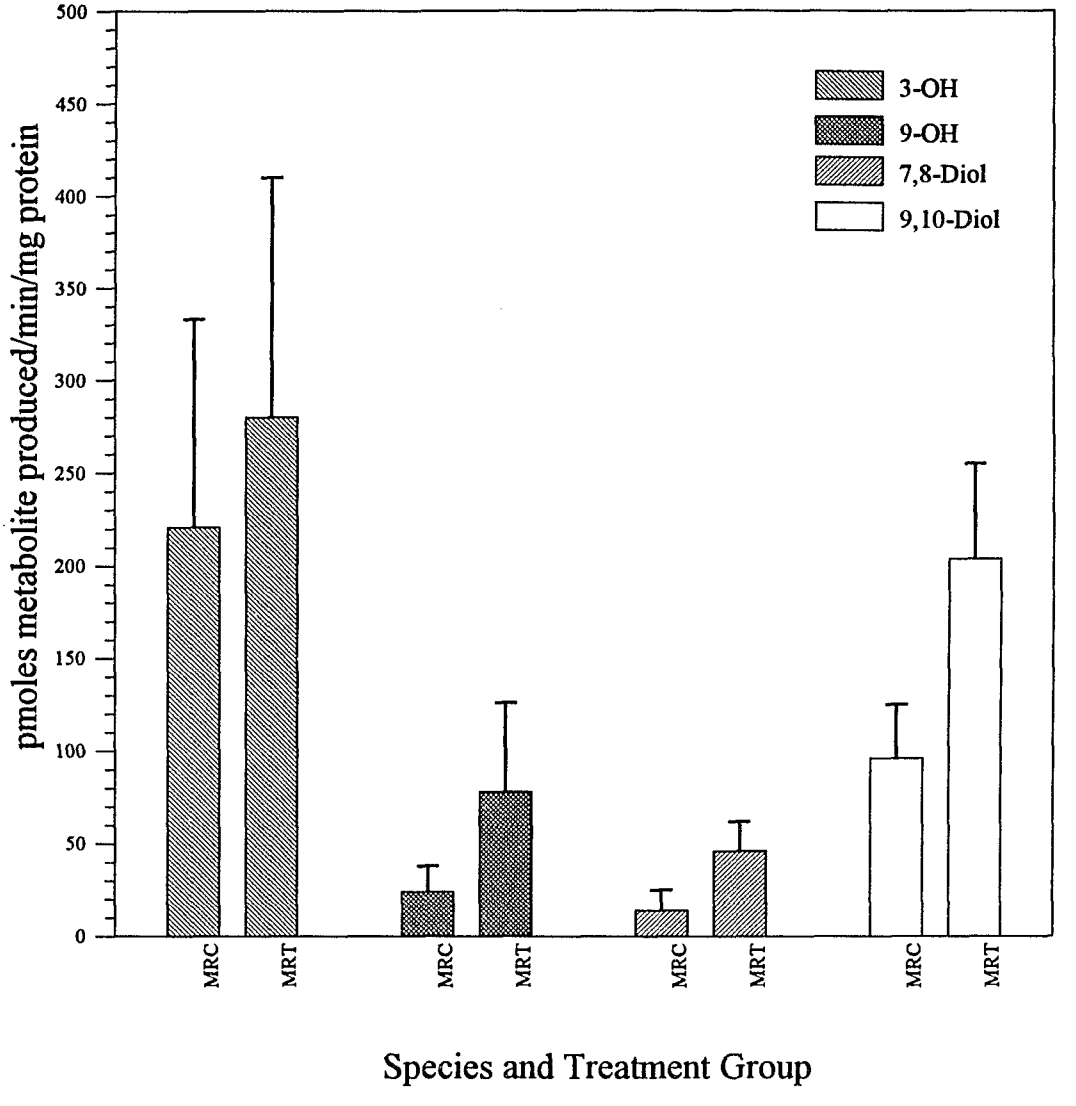

Figure 6-15 Hepatic microsomal production of benzo[a]pyrene metabolites in control (MRC) and BNF-treated (MRT) male rats. See Figure 6-6 for metabolite identifications.

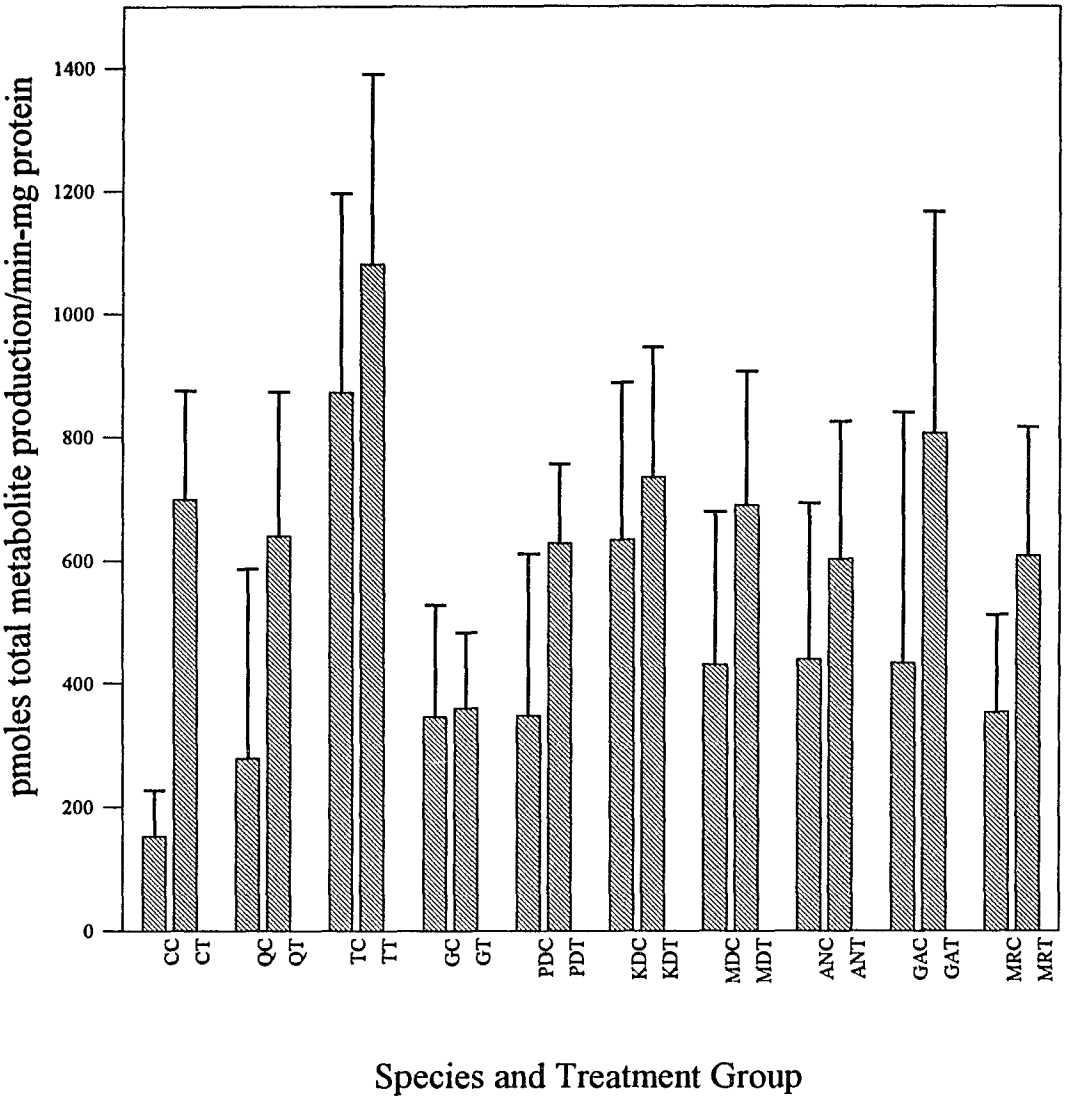

Figure 6-16 Hepatic microsomal production of total benzo[a]pyrene metabolites in control and BNF-treated avian species and male rats. Total metabolite production refers to the production of 3-OH, 9-OH, 7,8-diol, and 9,10-diol metabolites. See Figure 6-1 for species identifications.

observed following treatment with βNF. The constitutive capacity for ethoxyresorufin *O*-deethylation and the induction of this activity associated with βNF administration were similar for the avian species examined. In contrast, interspecies differences were noted in the capacity of the avian hepatic microsomal system to hydroxylate B[*a*]P and in the effect of βNF treatment upon total metabolite formation. Differences in metabolic profiles were also noted between the orders.

Constitutive EROD activity was comparable among all the species studied. When evaluated by order, however, greater basal activity was noted for the Anseriformes compared to the Galliformes. As seen in the rat, it appears that ethoxyresorufin has a high specificity for the βNF-induced hepatic microsomal cytochrome P-450 of the avian species. Following βNF treatment, significant increases in hepatic microsomal EROD activity were observed for each of the avian species examined. Increases based on specific activity were more pronounced than when based on turnover number. Induced levels observed for the birds were similar except for the Japanese quail which exhibited a significantly lower activity than all the other species except for the goose. Among the avian species, the induction response, based on EROD activity, was greatest for the chicken, intermediate for the Japanese quail, turkey, goose, Pekin duck, and Khaki Campbell duck and lowest for the Muscovy duck. Evaluated on the basis of order, the Galliformes demonstrated an induction of activity following βNF that was twice that seen for the Anseriformes order. In comparison of the major and minor species, the most notable finding was a significant difference

in the EROD activity between the treatment groups of the chicken and Japanese quail and the turkey and Japanese quail. Other significant differences noted were between the minor avian species.

Constitutive benzo[*a*]pyrene hydroxylase activity (based on the *in vitro* production of 3-OH B[*a*]P, 9-OH B[*a*]P, B[*a*]P 7,8-diol, and B[*a*]P 9,10-diol) was comparable for the Galliformes and Anseriformes orders. However, significant differences in basal activity were observed between the major species and between the major and minor species of these orders.

Quantitative as well as qualitative differences in B[*a*]P metabolism were observed following βNF treatment. Induction of B[*a*]P metabolism was observed in some avian species while others experienced no change or an actual decrease in metabolite formation. These differences were noted between the major species as well as between the major and minor species. In addition, greater AHH activity was observed in the Galliformes compared to the Anseriformes following βNF.

In general, the metabolic profiles produced by the Galliformes species were similar. However, the characteristics of B[*a*]P metabolism in the quail were more like those of the chicken than those of the turkey. Qualitative and quantitative differences in B[*a*]P metabolism were noted between the Anseriformes species. In general, the patterns of B[*a*]P metabolism demonstrated for the duck species were similar. The

goose however, demonstrated profiles of metabolite formation that were different from those observed for the ducks. In comparison of the Galliformes and Anseriformes, the patterns of B[*a*]P metabolism observed for the chicken, Japanese quail, and turkey were different from those observed for the Pekin duck, Khaki Campbell duck, Muscovy duck, and goose. However, 3-OH B[*a*]P was the primary metabolite produced by all the species studied. Interspecies and interorder differences in product distributions were for the 9-OH B[*a*]P, B[*a*]P 7,8-diol, and B[*a*]P 9,10-diol metabolites.

In comparison to the rat, the avian species exhibited comparable B[*a*]P hydroxylase activity except for the turkey and Khaki Campbell duck which exhibited greater activity (based on turnover number). Patterns of metabolite formation in the rat were qualitatively and quantitatively dissimilar to those of the avian species.

6.3 Summary and Conclusions

6.3.1 Approval of Minor Use Avian Drugs

This study has served to provide information on the cytochrome P-450-dependent metabolic capacity of seven major and minor food-producing avian species for the classical model substrate, benzo[*a*]pyrene, and to characterize the differences in metabolism of this compound between species. Characterization of this important biotransformation pathway has provided data for the estimation of the activity of the

avian hepatic mixed function monooxygenase system and for the relative rate of oxidation of structurally similar compounds in represented avian species.

The components of the hepatic microsomal system were demonstrated to be present at comparable levels in the Galliformes and Anseriformes species examined. This is an important finding since interspecies differences in metabolic capacity have been shown to be correlated with differences in cytochrome P-450 content and cytochrome P-450 reductase activity. The findings of this study indicate that there are interspecies differences in the capacity of the avian hepatic cytochrome P-450 system to hydroxylate B[*a*]P. Significant quantitative differences in constitutive activity were observed between the major species and between major and minor species. The most notable difference observed was the high level of constitutive B[a]P hydroxylase activity demonstrated by the turkey compared to the other avian species. This finding suggests that drugs subject to biotransformation pathways involving polynuclear hydroxylation may be metabolized at a comparatively faster rate in turkeys than in other avian species. Similar metabolic profiles were demonstrated by the major and minor species and, in general, by the minor duck species. Of the birds studied, the characteristics of B[a]P metabolism were most different for the goose. This finding suggests, that for drugs similar in structure to the model compound B[*a*]P, it may not be appropriate to base predictions of drug fate in the goose on biotransformation data obtained in the chicken and turkey (major species). Qualitative and quantitative differences in the microsomal hydroxylation of B[*a*]P were observed between the

major species and between major and minor species following treatment with βNF. Differences were also noted between the orders. This variation in response is noteworthy from the standpoint that food-producing avian species are routinely exposed to a variety of chemicals that may affect cytochrome P-450-dependent activities and thus the biotransformation of administered therapeutic agents.

The results of this study further demonstrate that there are marked differences in the characteristics and metabolic capacity of the avian and rat hepatic cytochrome P-450 systems. Consequently, it should be re-emphasized that predictions concerning drug metabolism in avian species should not be based on biotransformation data generated in the rat or other mammalian species.

This work has provided an important contribution to the comparative study of xenobiotic metabolism in domestic avian species by characterizing the components of the hepatic cytochrome P-450 system in seven major and minor species and by examining the metabolic oxidative capacity of these species though the development of metabolite profiles for a model substrate. It is anticipated that the results of this study will serve to support the interpretation of data submitted to the FDA for the purpose of obtaining approval for drug use in economically minor food-producing species such as the Japanese quail, Pekin duck, Khaki Campbell duck, Muscovy duck and goose.

6.3.2 Domestic Avian Species as Sentinels in Ecotoxicological Studies

The results of the present study clearly indicate that the avian hepatic microsomal cytochrome P-450 mixed function monooxygenase system of selected domestic species is inducible by β-naphthoflavone, a classical inducer belonging to an environmentally relevant class of compounds, the PAHs. The most consistent response observed following exposure to this compound was for the *O*-deethylation of ethoxyresorufin. Significant increases in this cytochrome P-450-dependent activity were observed for all seven species studied. This finding indicates that the avian hepatic cytochrome P-450 system of domestic avian species may be inducible by PAHs present in the environment and that the induction of EROD activity in these species may be useful as a biomarker of exposure to this class of compounds.

In contrast, the observed effects of βNF administration on the other microsomal parameters examined varied between the species. However, two species, the chicken and Pekin duck, demonstrated increases in microsomal protein, cytochrome P-450 content, and B[*a*]P hydroxylase activity following βNF treatment. Therefore, it is possible that the induction of these parameters, particularly AHH activity, may be used as biomarkers of environmental chemical exposure in biomonitoring programs involving these species. The most dramatic response to βNF treatment was observed for the hepatic microsomes of the chicken. Not only were increases observed for all the parameters examined (excluding cytochrome *c* reductase), but the chicken demonstrated the greatest increases in EROD and AHH activity of all the avian

species studied. This finding suggests, that for the species examined, the chicken may be the best species to use as a sentinel for the assessment of exposure to PAHs present in the environment.

In situ assessments, utilizing test organisms, may be used to evaluate the potential impact of environmental contamination on human health or the health of indigenous populations (Sandhu and Lower, 1989). In light of the findings of this study, it is reasonable to propose that domestic avian species have potential application as sentinels of environmental chemical exposure by the utilization of these species in *in situ* assessments. Domestic species are readily available, inexpensive and relatively easy to maintain thus making them suitable test organisms for most sites of environmental concern.

In addition, based on the significant increases in EROD activity seen for the turkey, quail, Khaki Campbell duck, Muscovy duck and goose following βNF administration, it can be concluded that indigenous populations of turkeys, ducks, geese and quail may have potential use as sentinel species for the assessment of environmental contamination.

Futhermore, based on the demonstrated changes in B[*a*]P metabolite distributions associated with βNF exposure in these species, it may be possible, with further research, to utilize induction of the MFO system as a nondestructive biomarker of

chemical exposure. This concept is based on the administration a nontoxic model compound, via the use of bait, to indigenous avian populations and the subsequent analysis of excreted metabolites to determine if metabolic pathways have been altered due to induction of the hepatic cytochrome P-450 system.

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APPENDIX: SUPPORTING FIGURES FOR METHODS

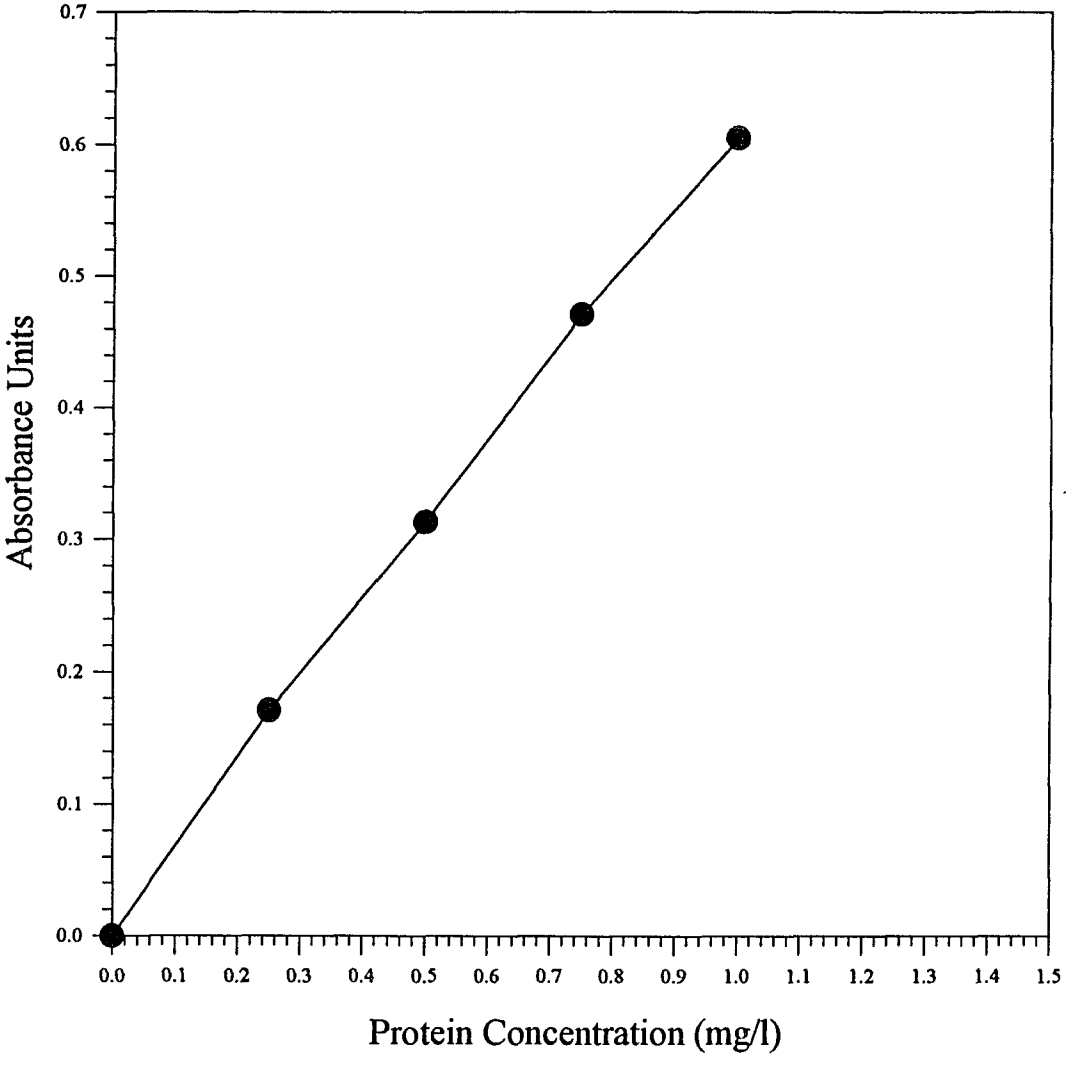


Figure A-1 Standard curve for the analysis of protein concentration using bovine serum albumin (BSA).

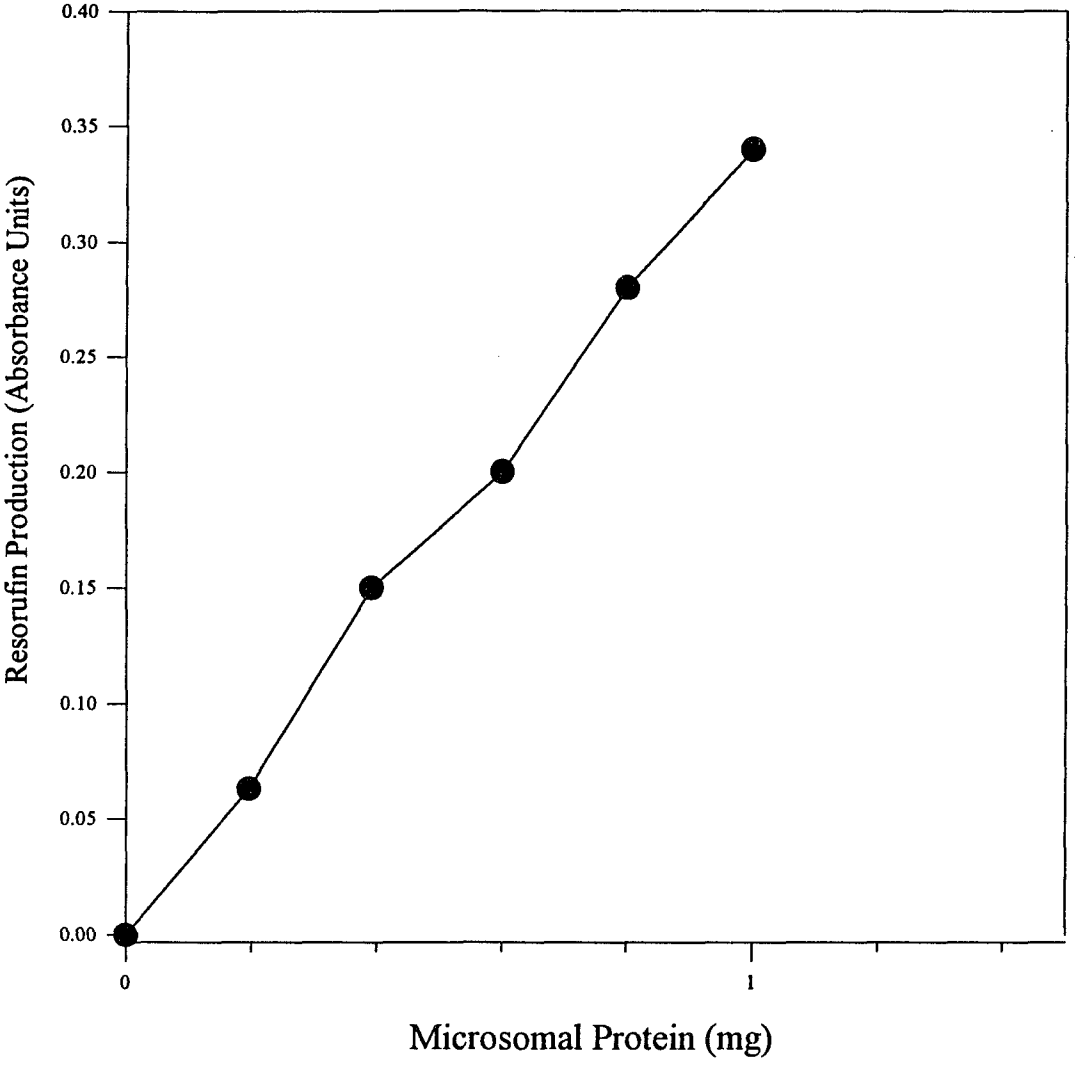


Figure A-2 Effect of hepatic microsomal protein concentration on resorufin production for the control Pekin duck.

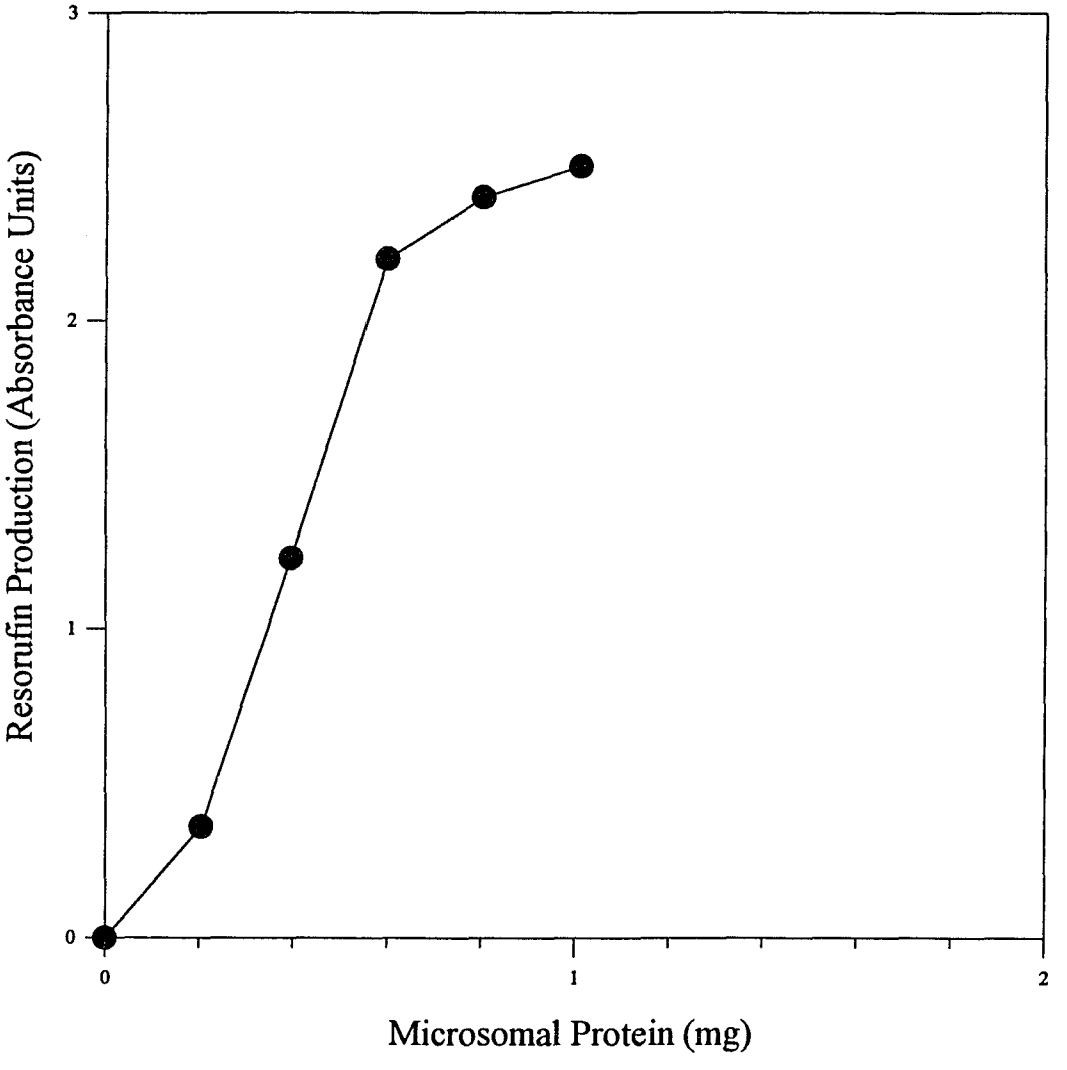


Figure A-3 Effect of hepatic microsomal protein concentration on resorufin production for the treated Pekin duck.

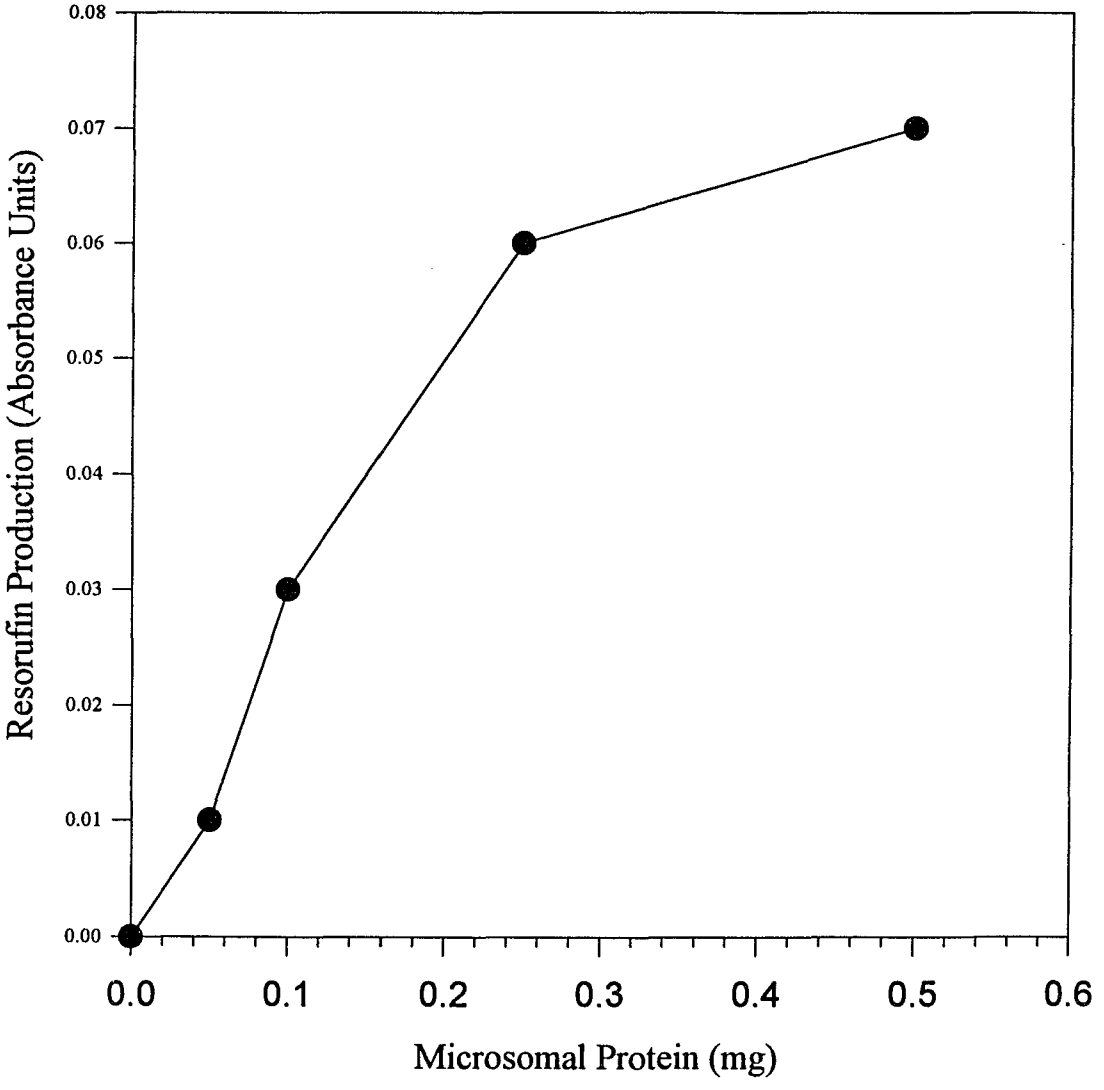


Figure A-4 Effect of hepatic microsomal protein concentration on resorufin production for the control male rat.

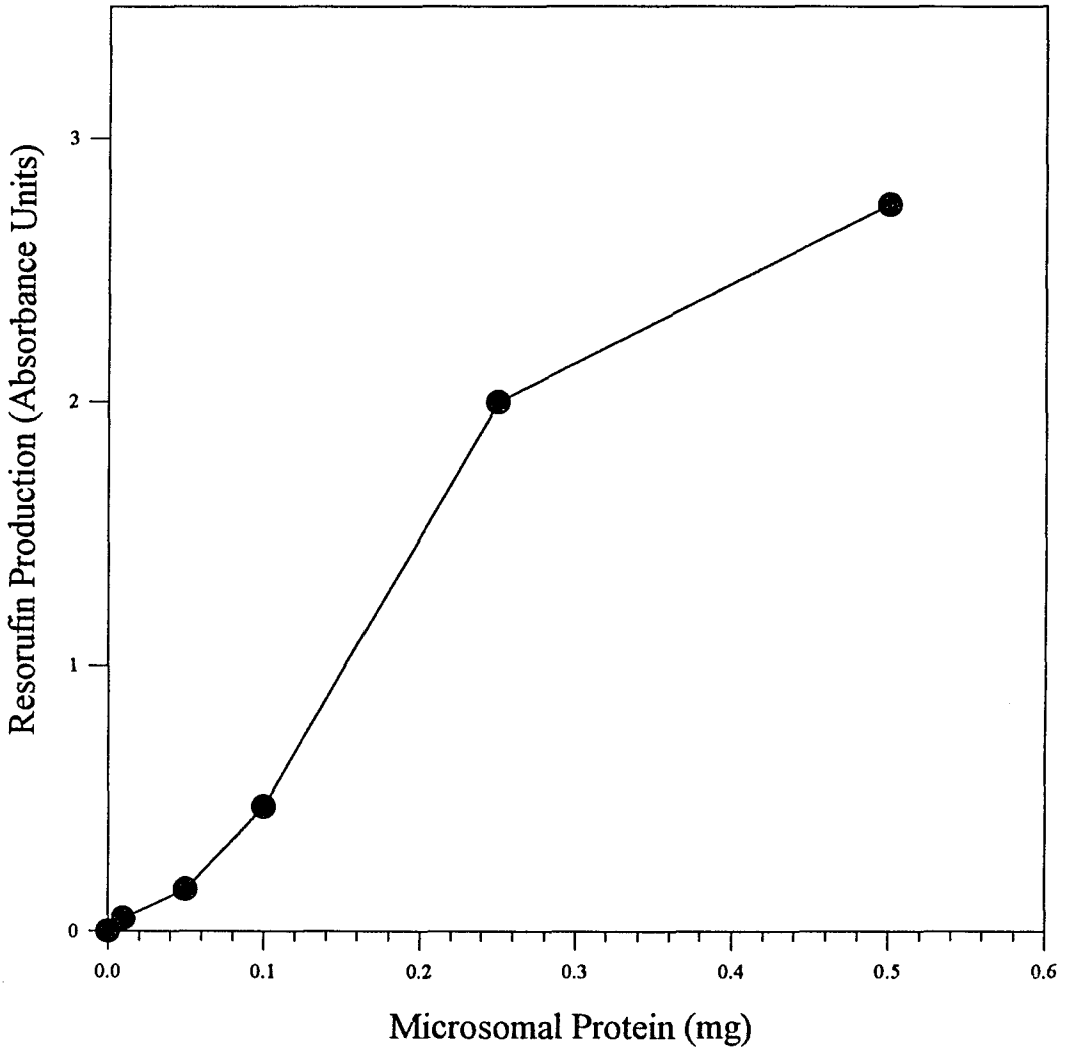


Figure A-5 Effect of hepatic microsomal protein concentration on resorufin production for the treated male rat.

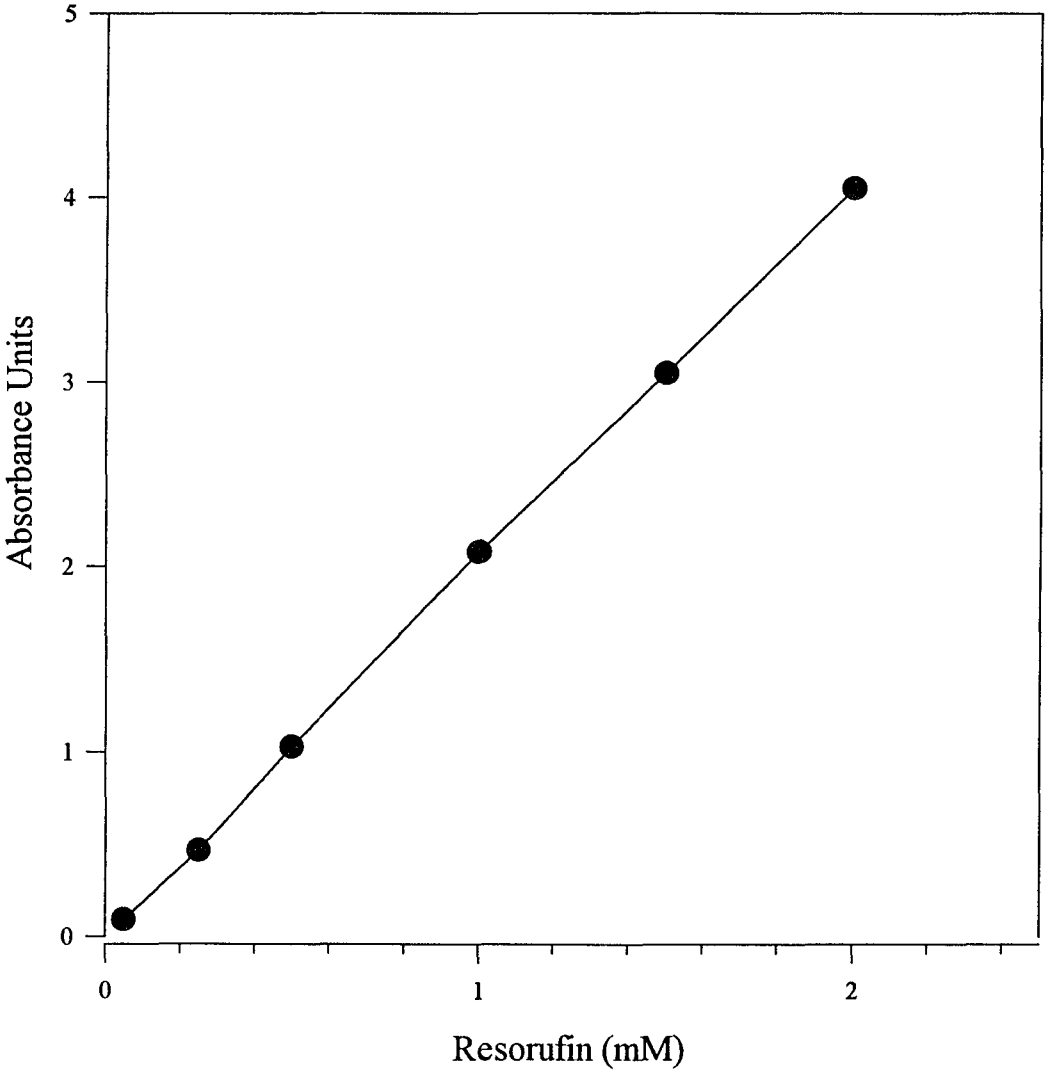


Figure A-6 Resorufin standard curve for the determination of ethoxyresorufin O-deethylase activity.

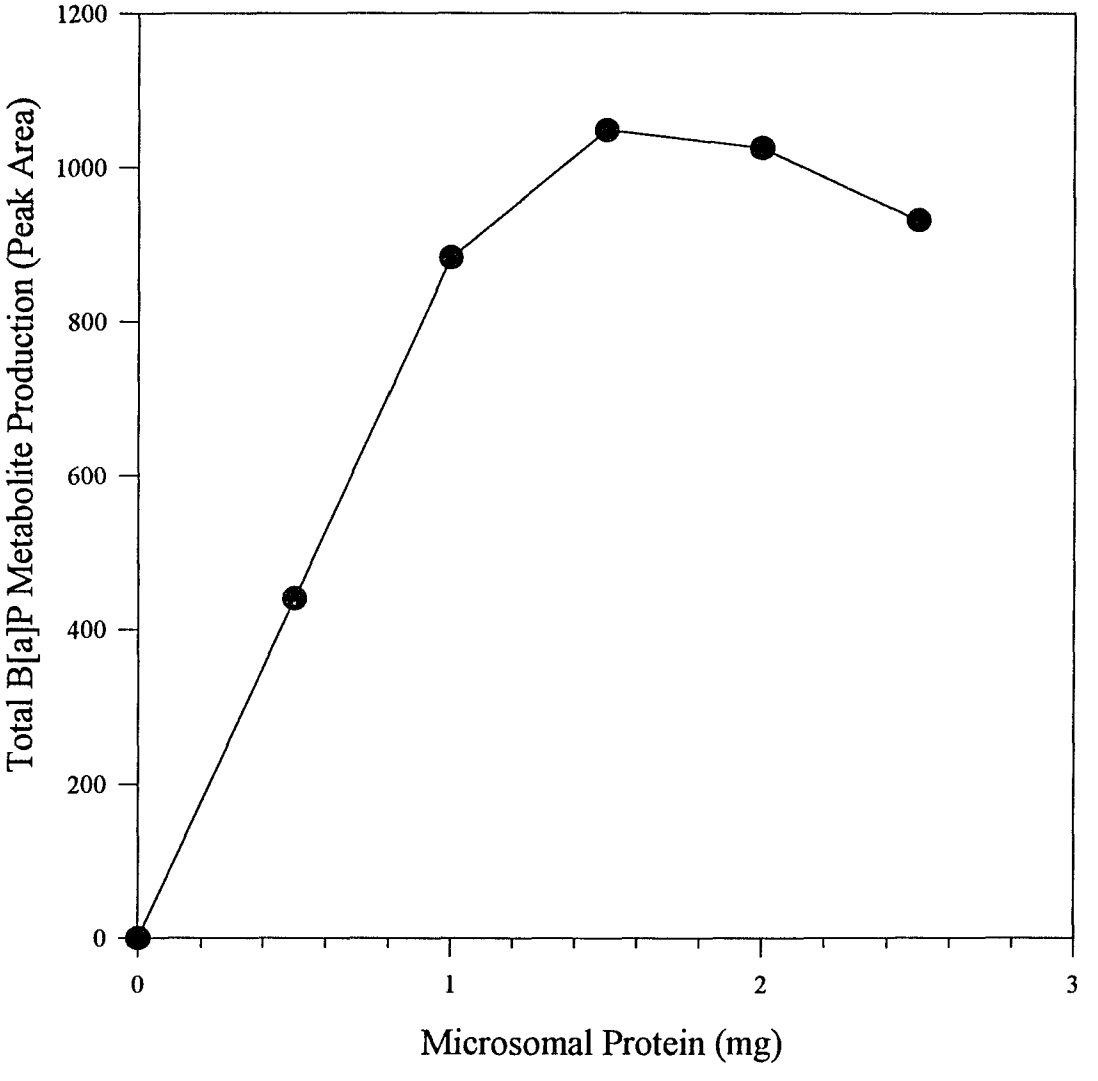
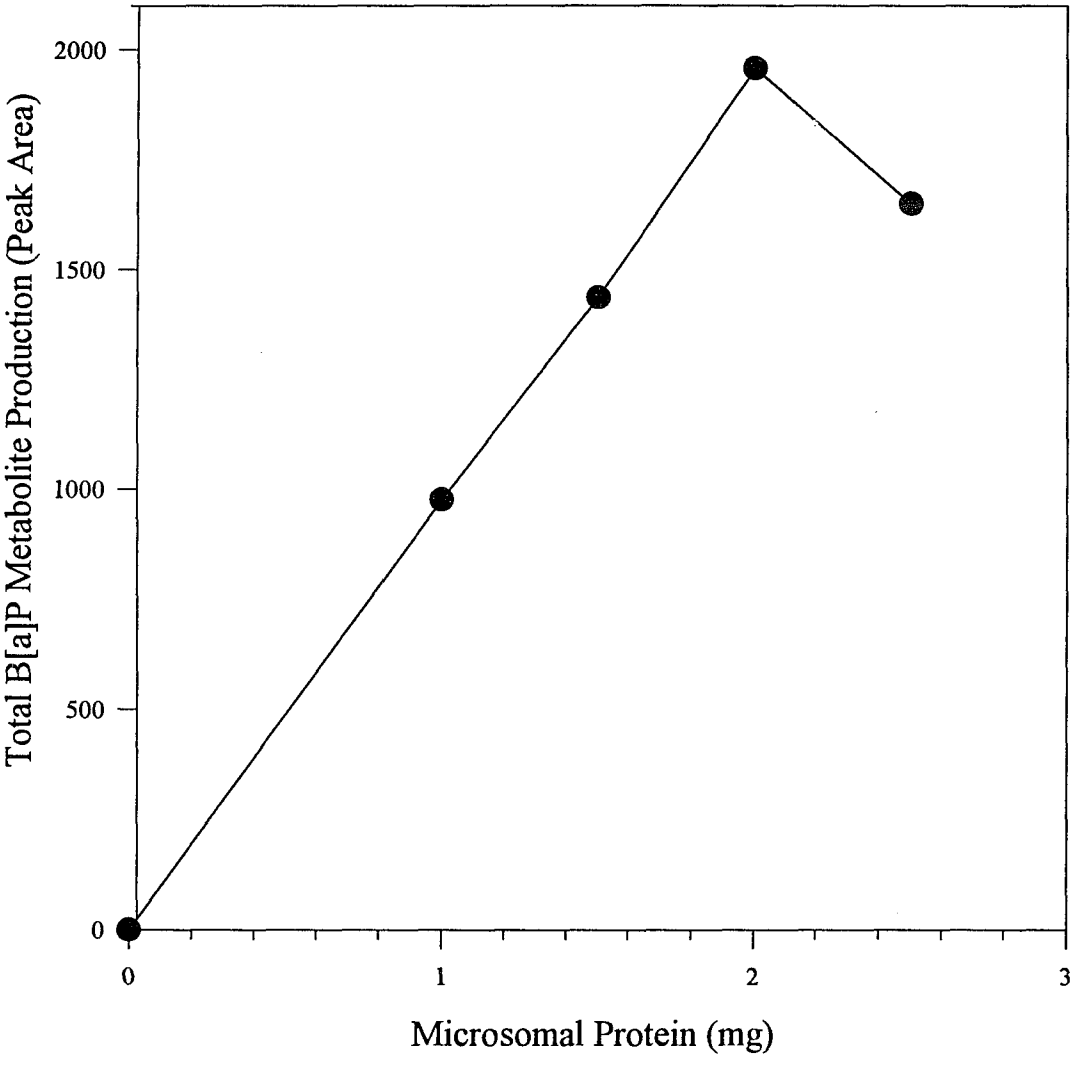


Figure A-7 Effect of hepatic microsomal protein concentration on benzo[a]pyrene metabolite production for the control chicken.



FigureA-8 Effect of hepatic microsomal protein concentration on benzo[a]pyrene metabolite production for the BNF-treated chicken.

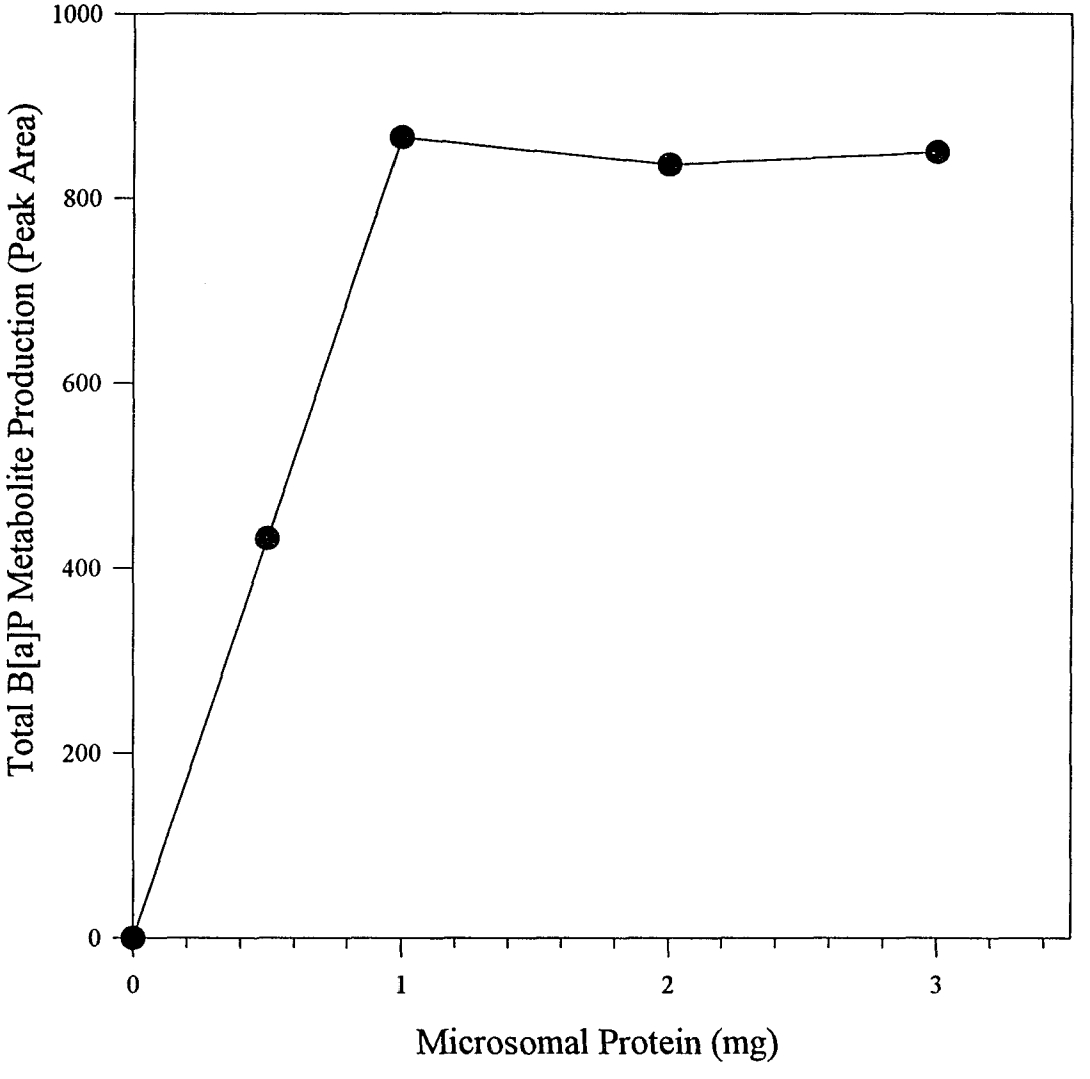


Figure A-9 Effect of hepatic microsomal protein concentration on benzo[a]pyrene metabolite production for the control rat.

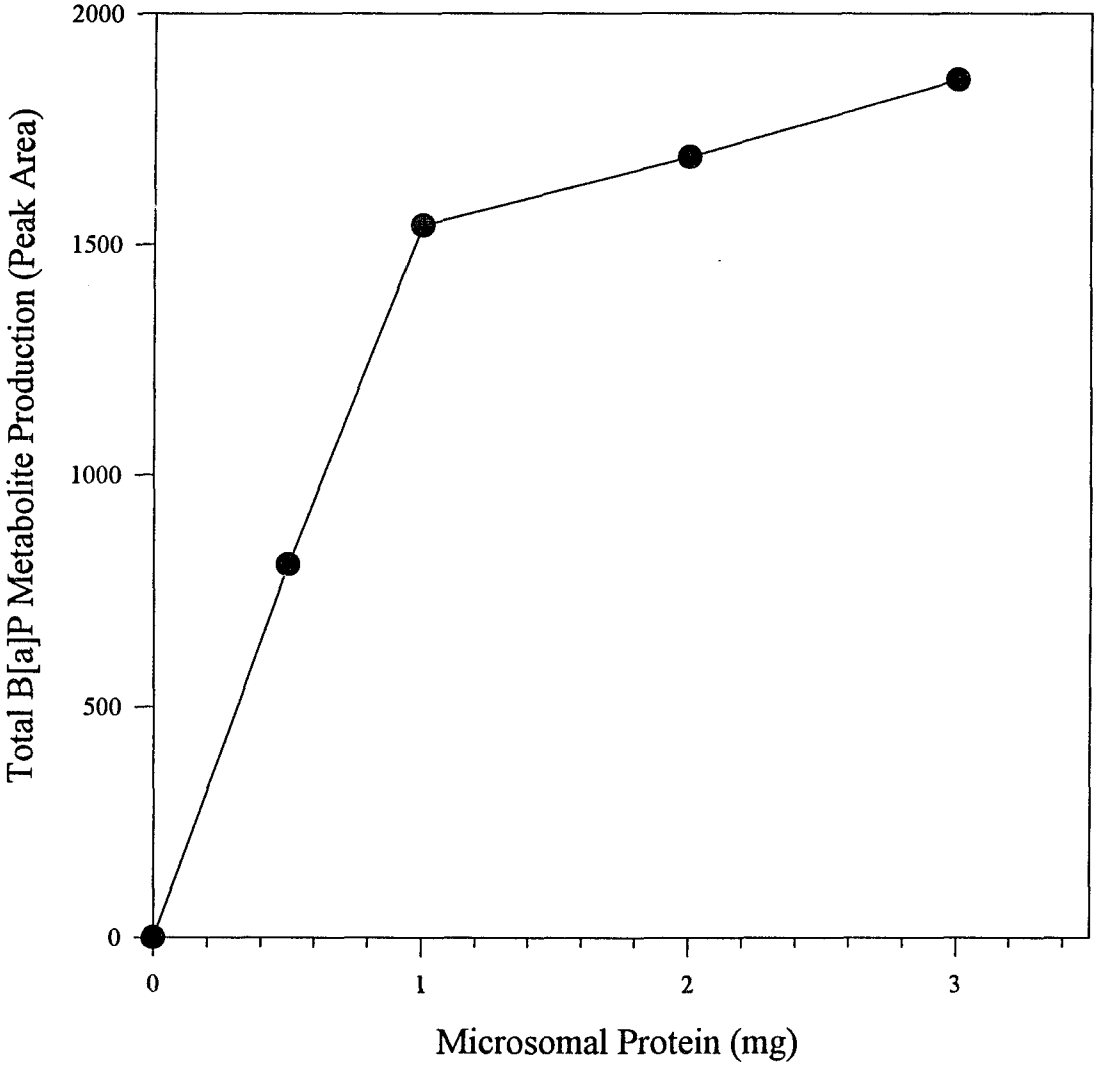


Figure A-10 Effect of hepatic microsomal protein concentration on benzo[a]pyrene metabolite production for the BNF-treated rat.

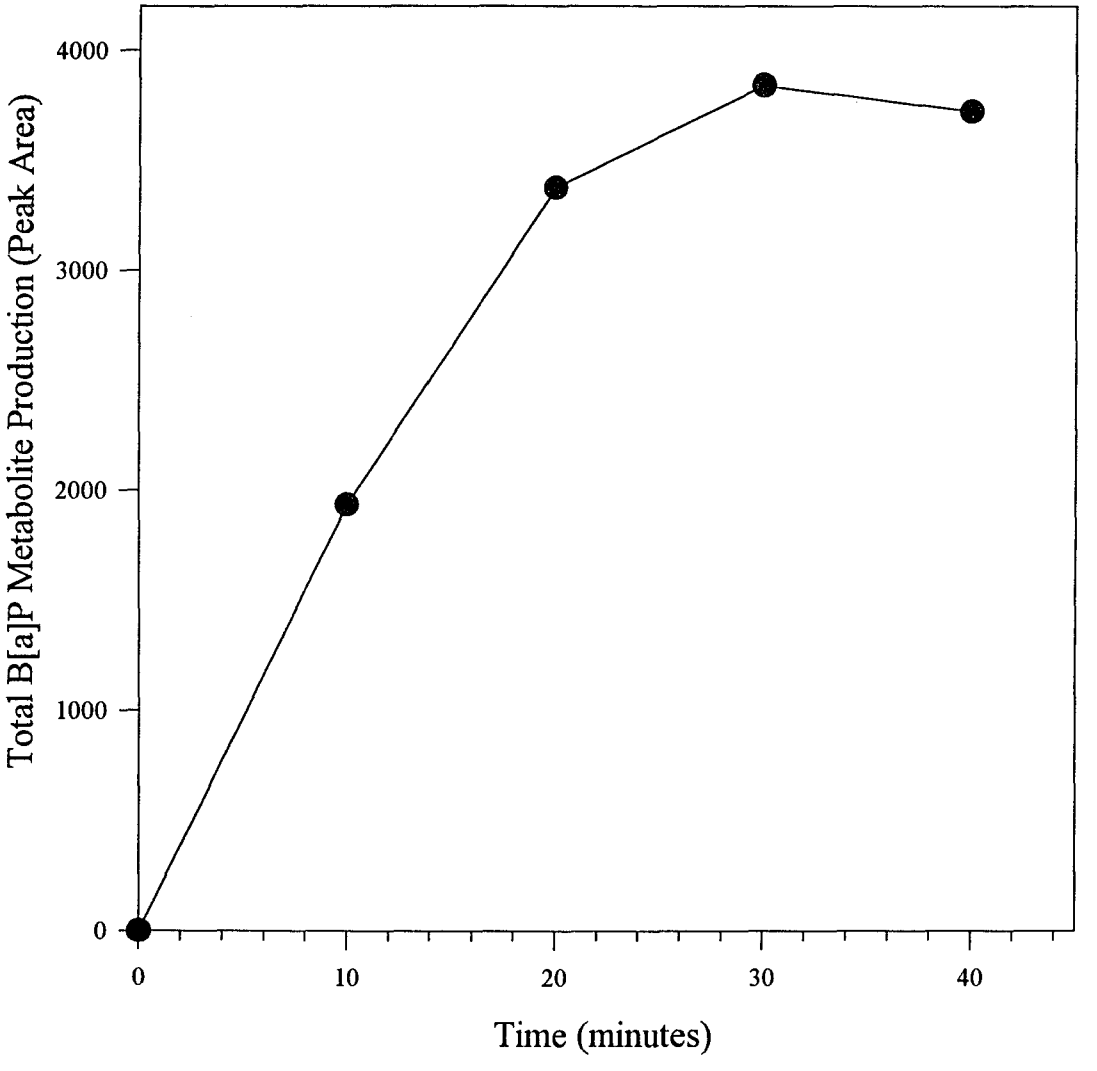


Figure A-11 Effect of incubation time on benzo[a]pyrene metabolite production for the BNF-treated chicken.

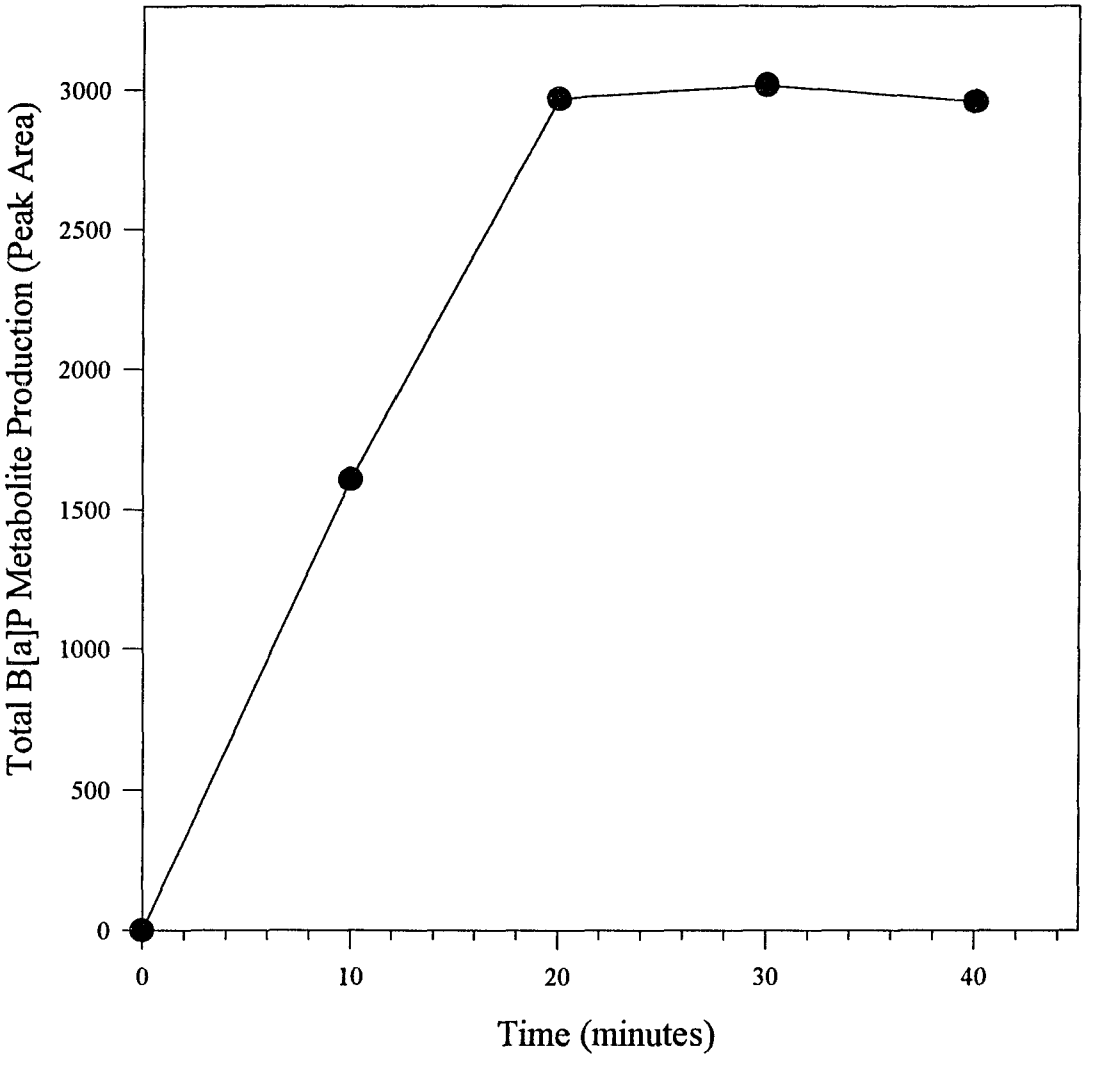


Figure A-12 Effect of incubation time on benzo[a]pyrene metabolite production for tne BNF-treated rat.

STANDARD CURVES FOR QUANTITATION OF BENZO[a]PYRENE AND METABOLITES

Quantitation of the metabolites was achieved through the use of external standard calculations. Standards were prepared from stock solutions of B[a]P and each of the metabolites. Peak areas were used to establish standard curves for each analyte by plotting the integration areas of the generated peaks.

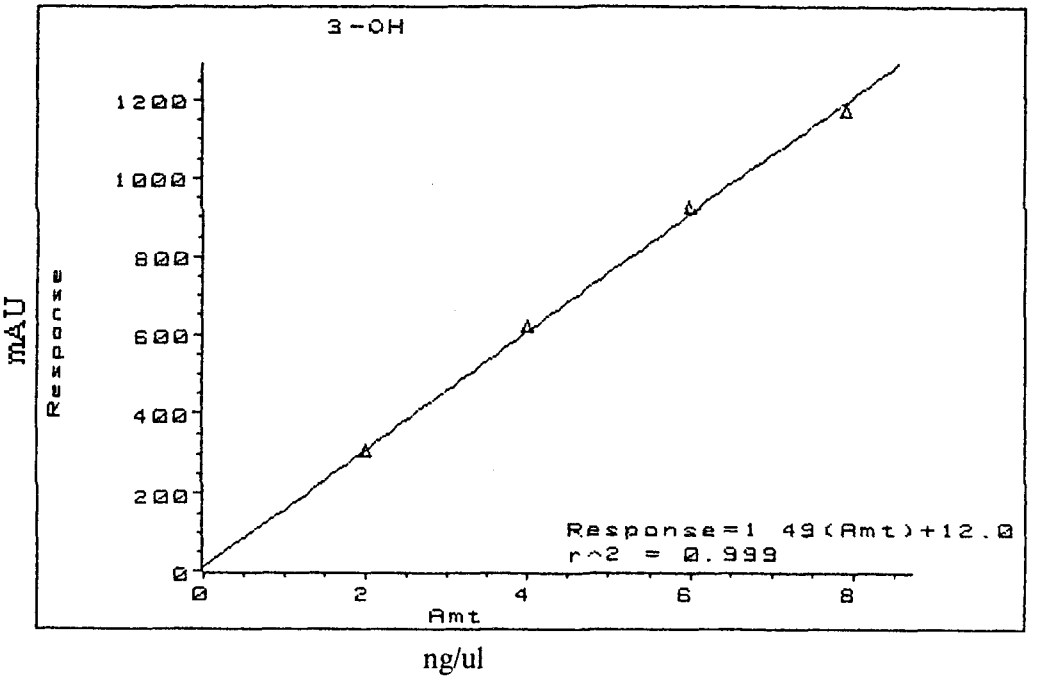


Figure A-13 Standard curve for 3-hydroxybenzo[*a*]pyrene.

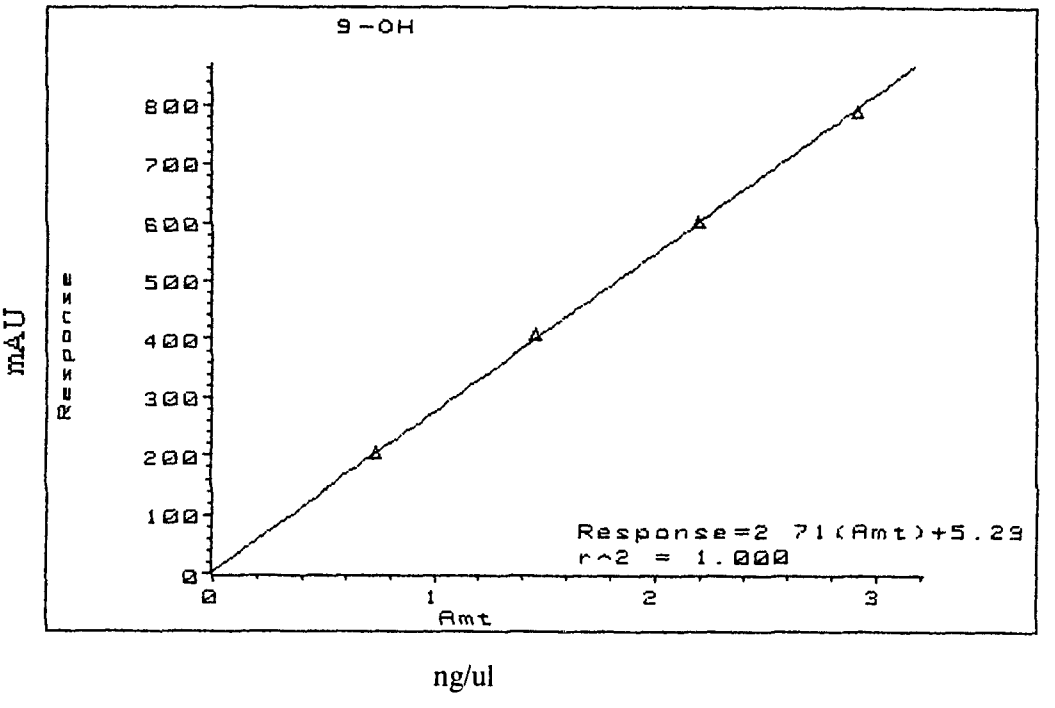


Figure A-14 Standard curve for 9-hydroxybenzo[*a*]pyrene.

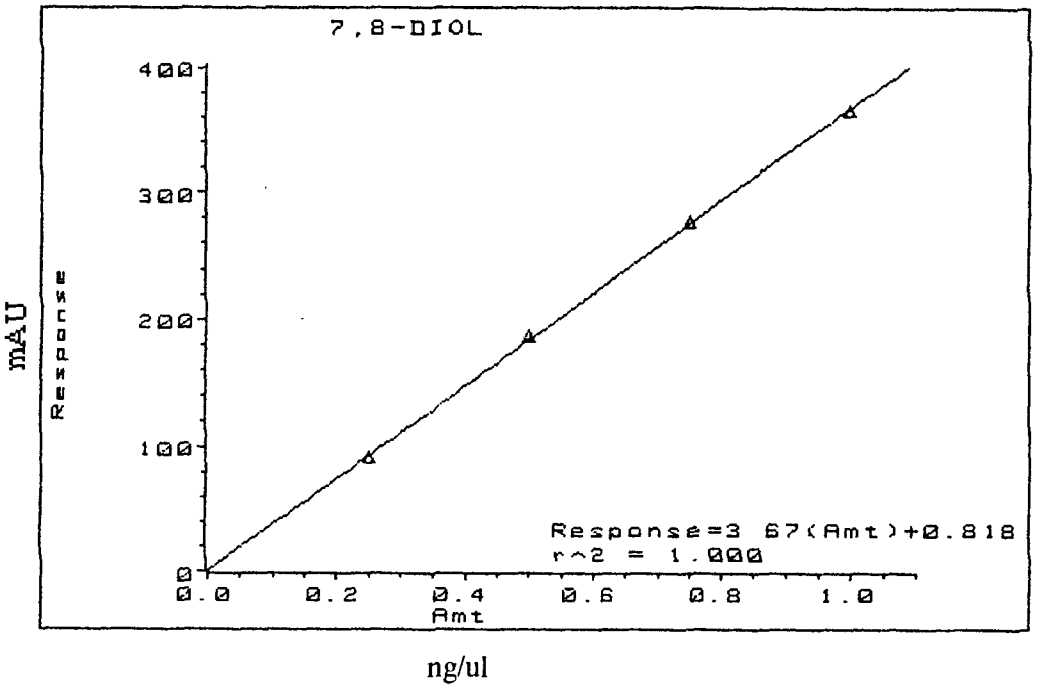


Figure A-15 Standard curve for benzo[*a*]pyrene-7,8-diol.

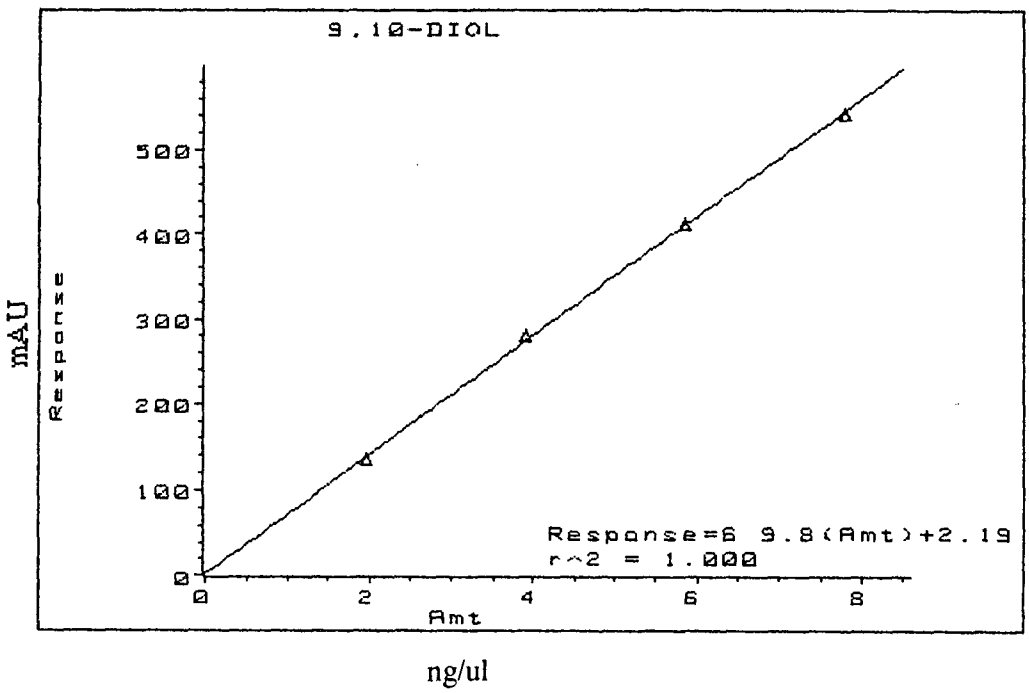


Figure A-16 Standard curve for benzo[a]pyrene-9,10-dihydrodiol.

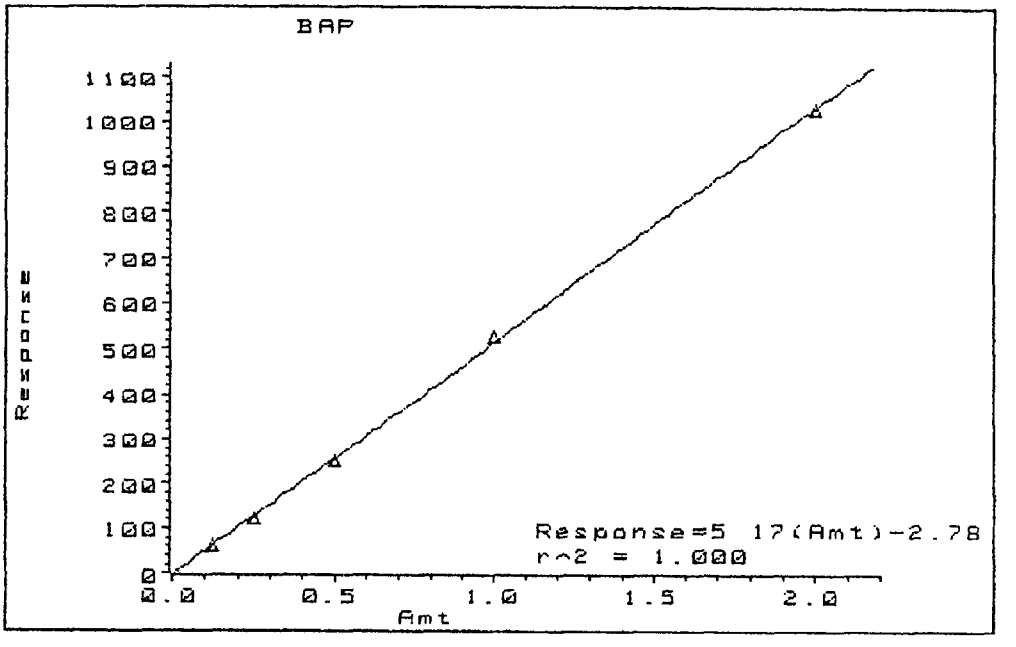


Figure A-17 Standard curve for benzo[*a*]pyrene.

METHOD BLANK CHROMATOGRAMS

A method blank was prepared for each analyte to determine if there were any components in the reaction mixture which might interfere with the identification and quantitation of the compounds interest. The method blank consisted of the reaction mixture without the microsomes and was spiked with B[*a*]P or one of the metabolites.

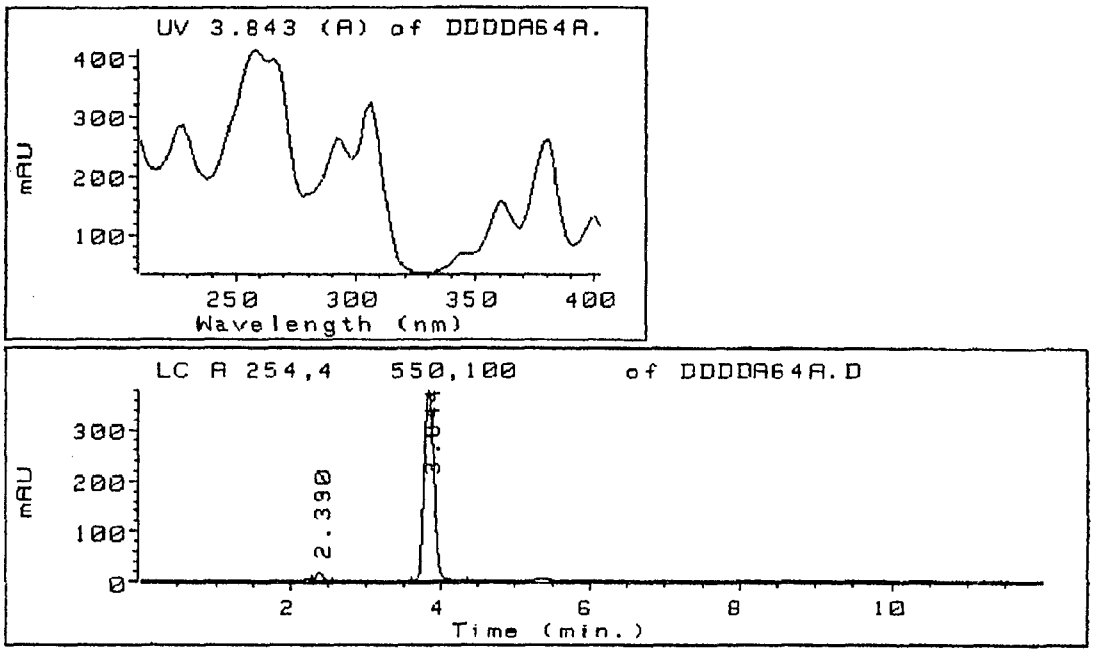


Figure A-18 Method blank chromatogram and UV spectrum for 3-hydroxybenzo[*a*]pyrene.

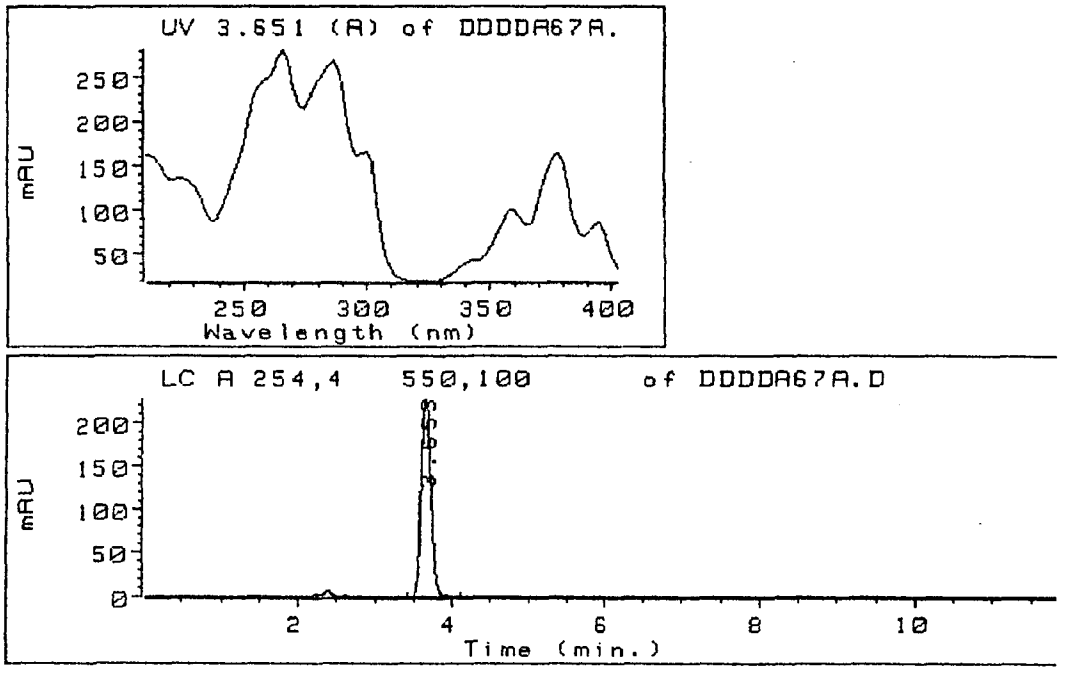


Figure A-19 Method blank chromatogram and UV spectrum for 9-hydroxybenzo[*a*]pyrene.

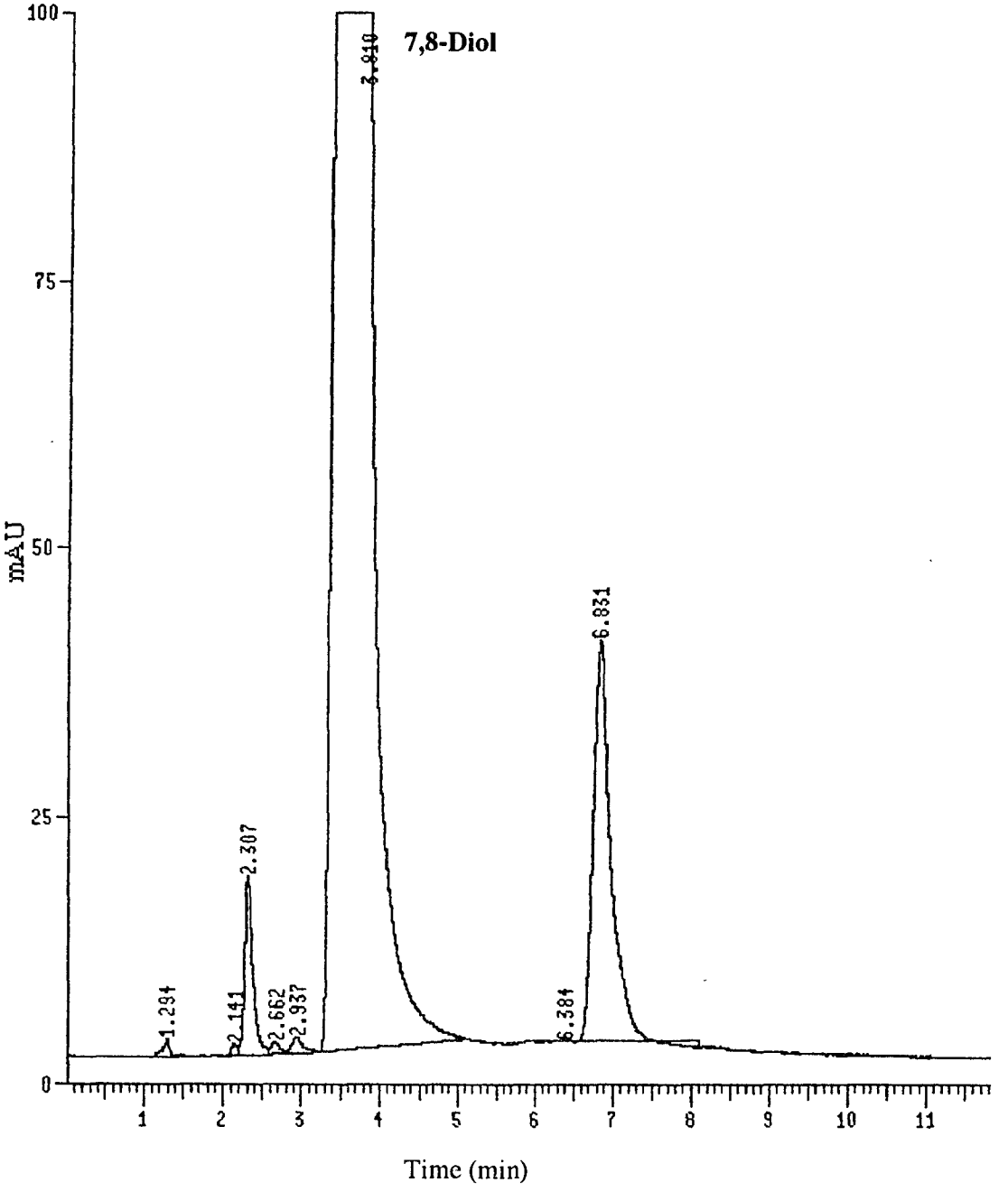


Figure A-20 Method blank chromatogram for benzo[*a*]pyrene 7,8-dihydrodiol.

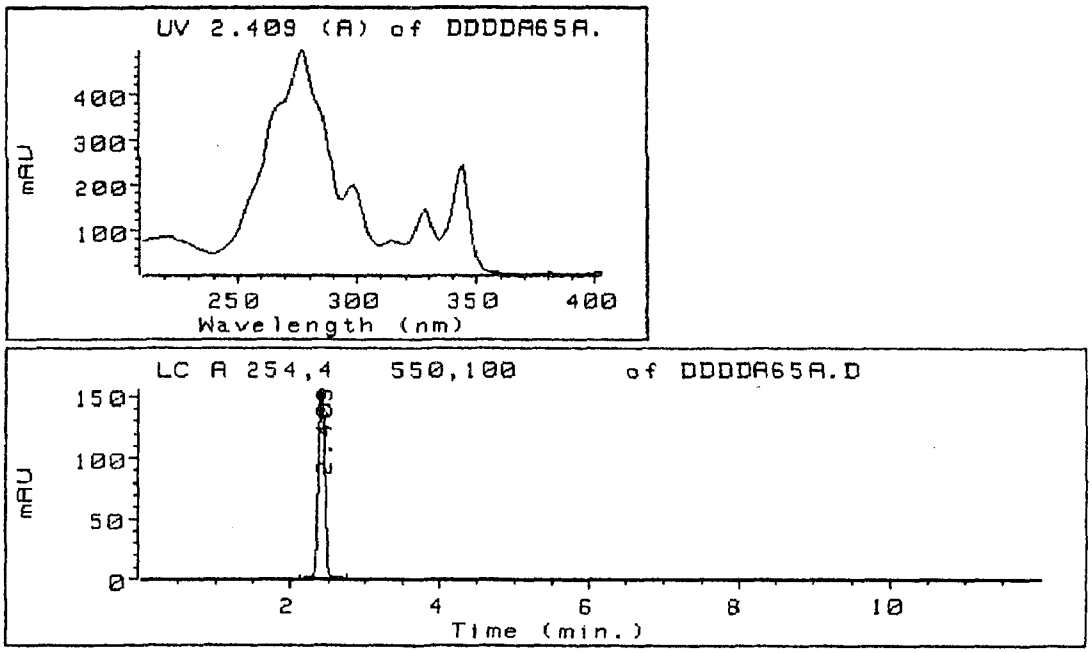


Figure A-21 Method blank chromatogram and UV spectrum for benzo[*a*]pyrene 9,10-dihydrodiol.

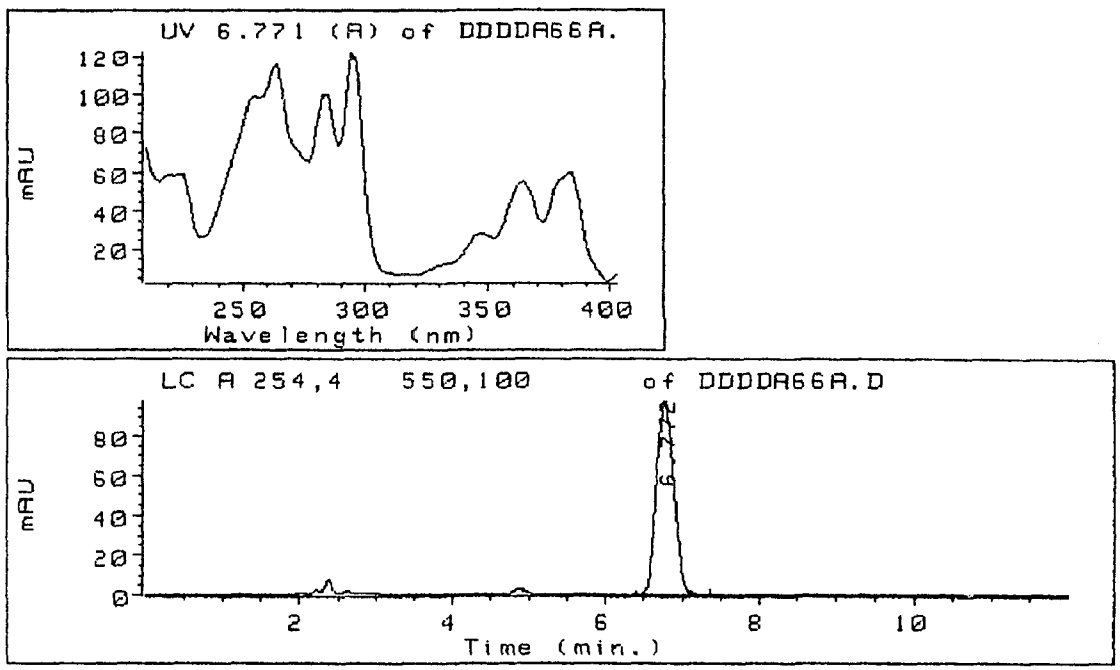


Figure A-22 Method blank chromatogram and UV spectrum for benzo[*a*]pyrene.

MICROSOMAL BLANK CHROMATOGRAMS

A microsomal blank was prepared for each species to determine if there were any microsomal components which may interfere with the identification and quantitation of the compounds of interest. The microsomal blank consisted of the reaction mixture without B[*a*]P.

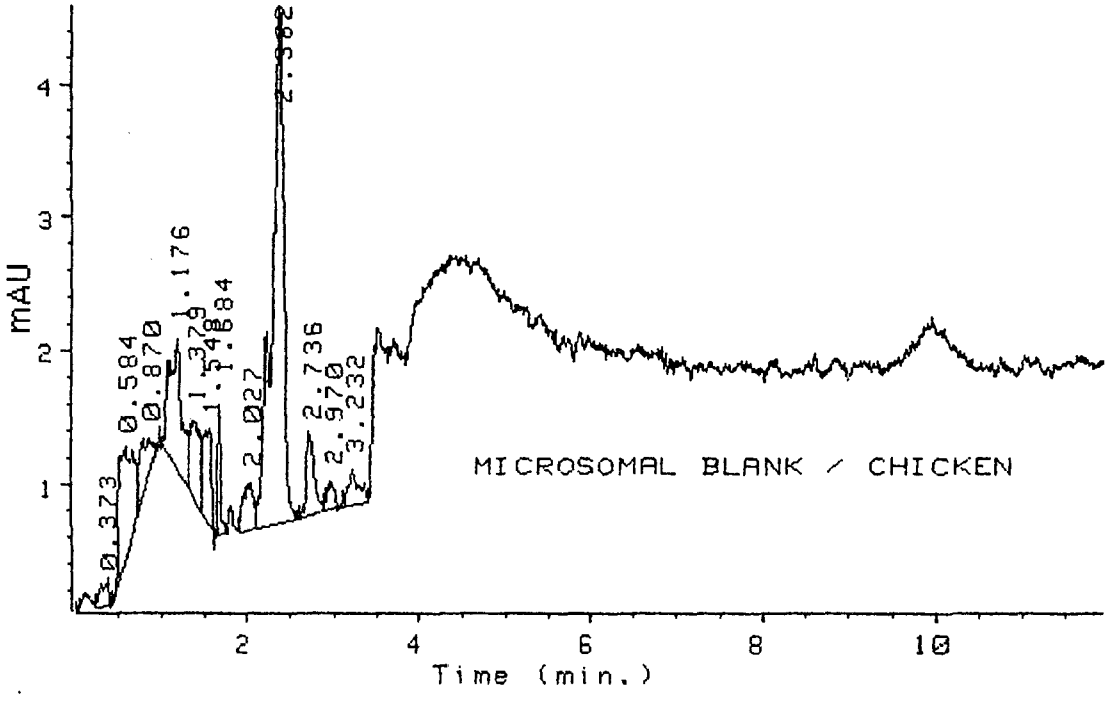


Figure A-23 Microsomal blank chromatogram for the chicken.

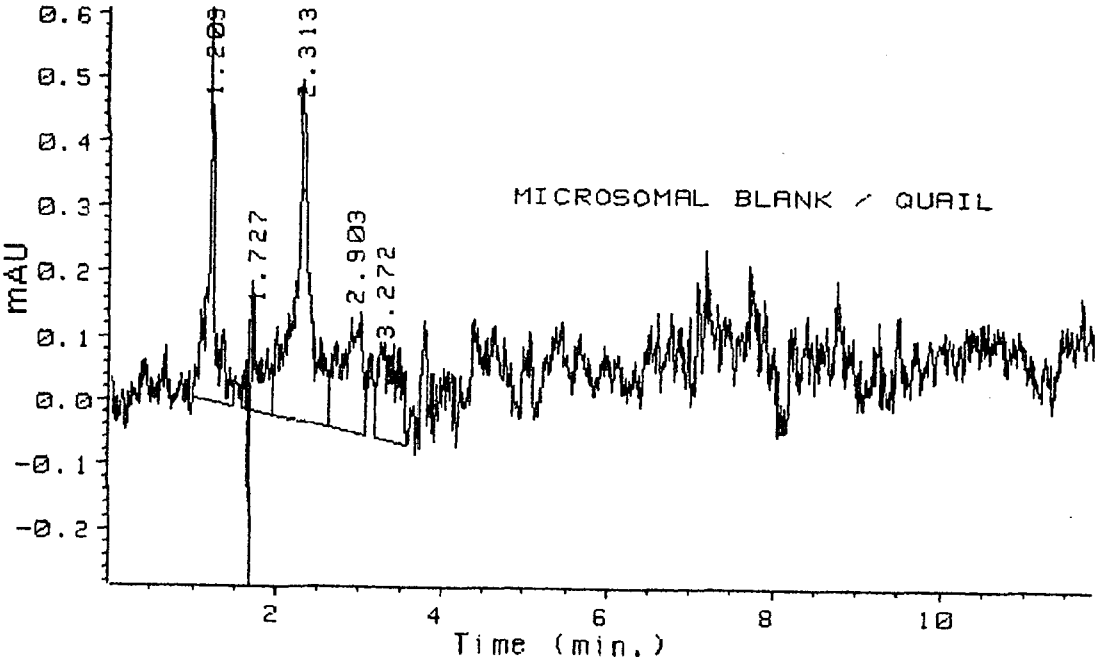


Figure A-24 Microsomal blank chromatogram for the Japanese quail.

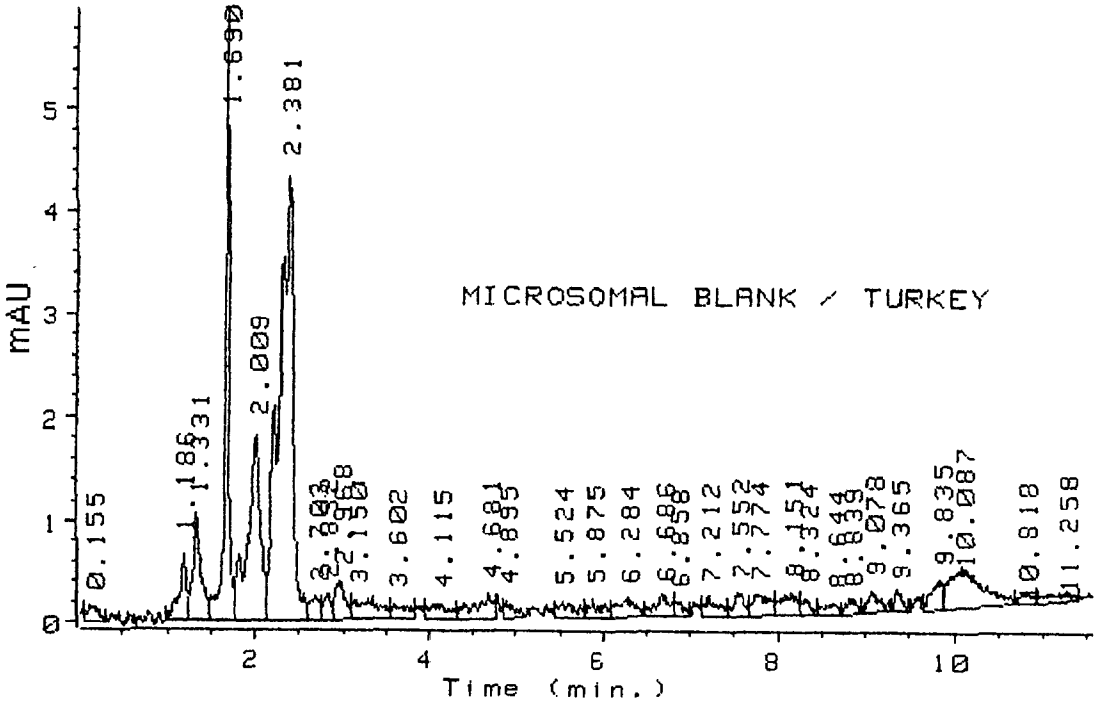


Figure A-25 Microsomal blank chromatogram for the turkey.

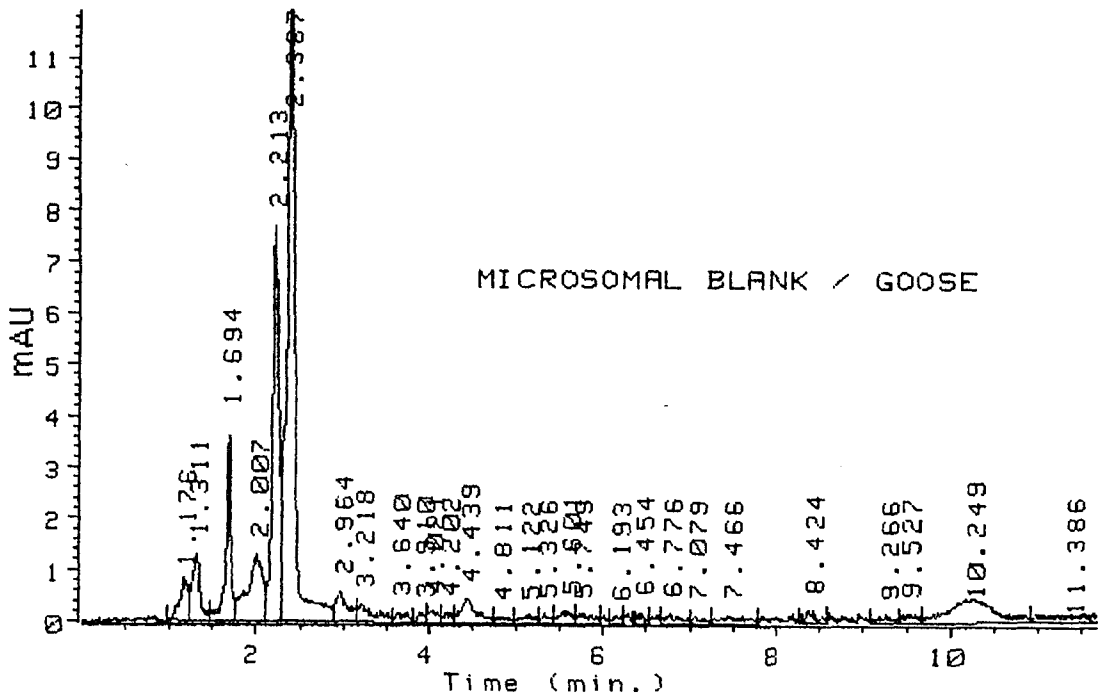


Figure A-26 Microsomal blank chromatogram for the goose.

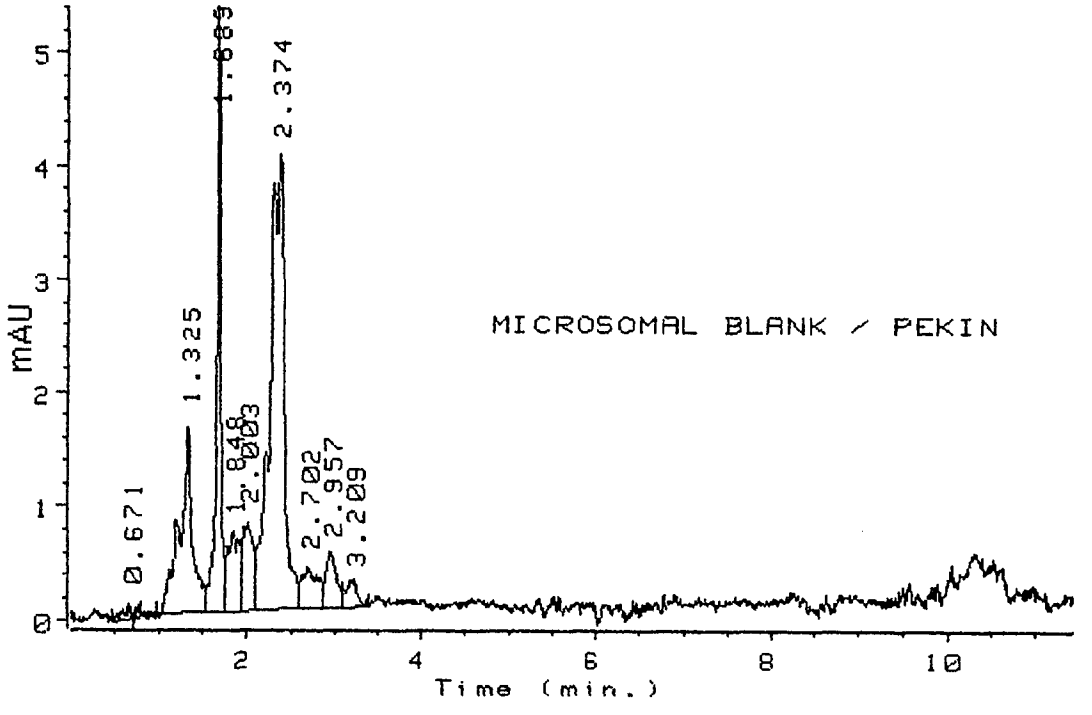


Figure A-27 Microsomal blank chromatogram for the Pekin duck.

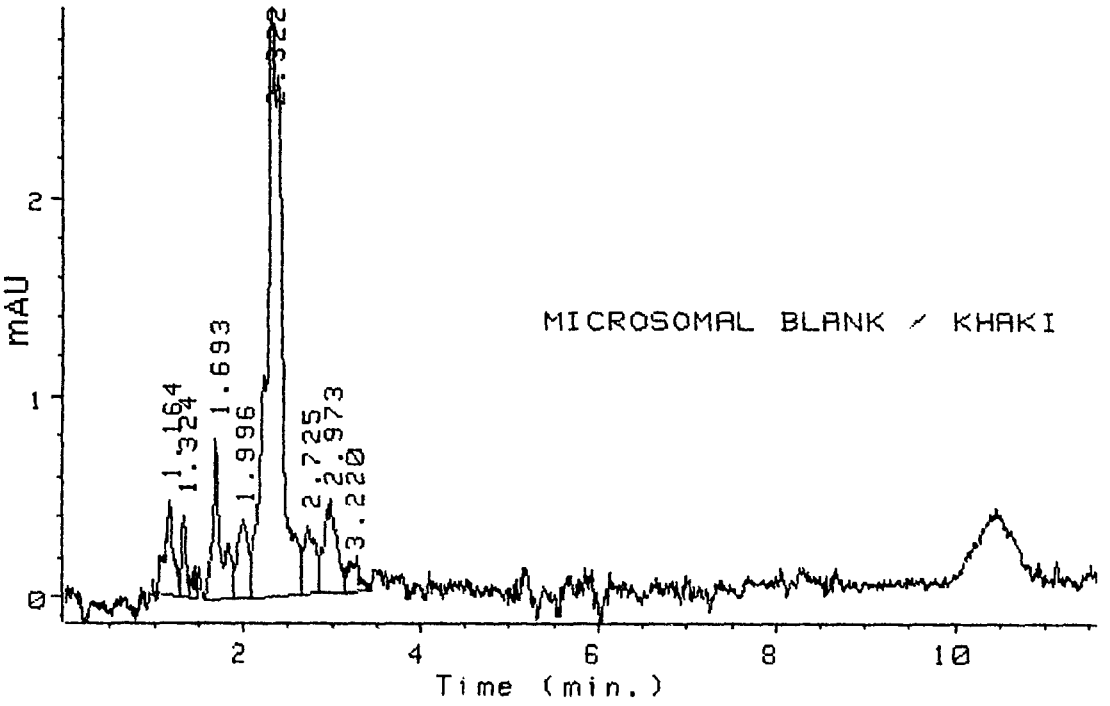


Figure A-28 Microsomal blank chromatogram for the Khaki Campbell duck.

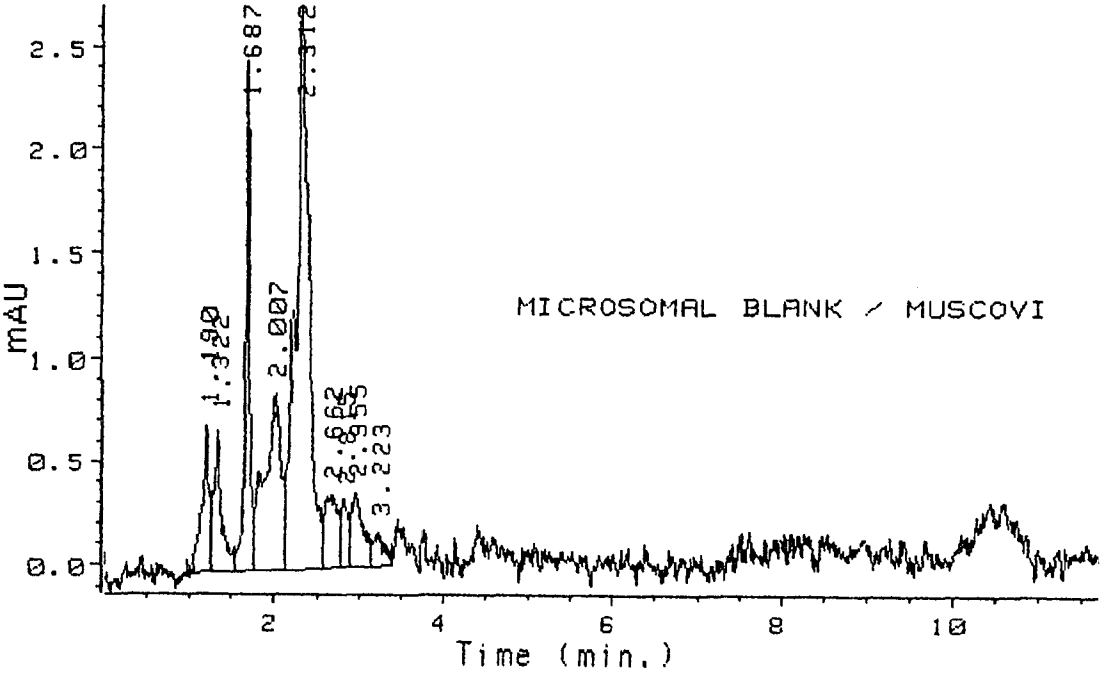


Figure A-29 Microsomal blank chromatogram for the Muscovy duck.

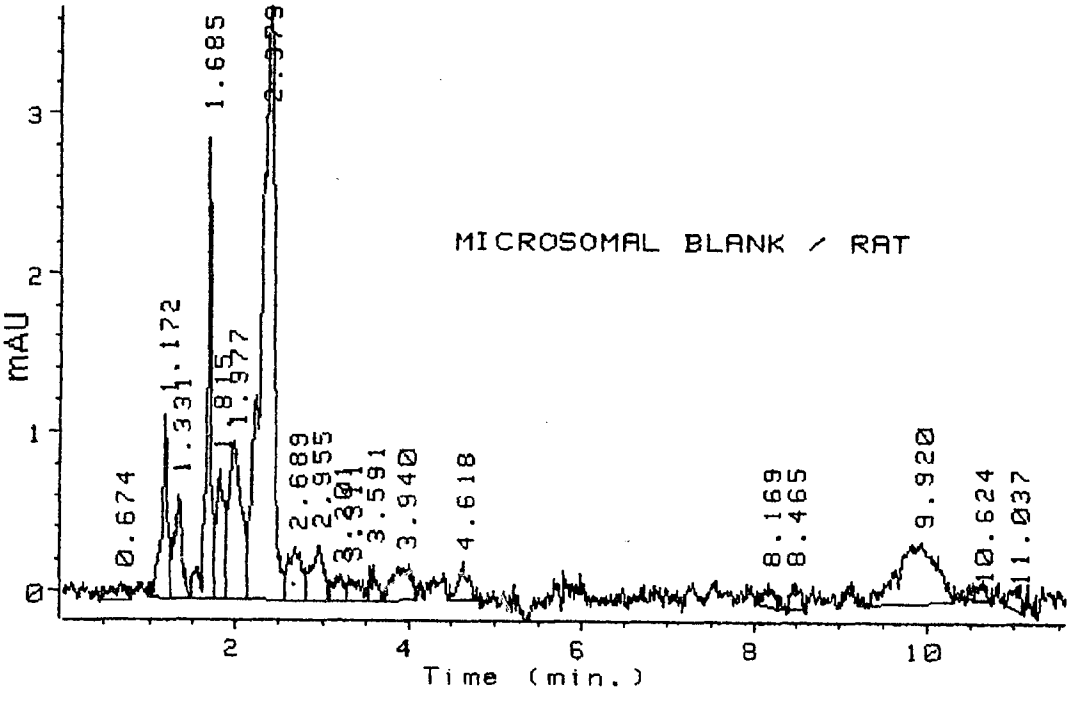


Figure A-30 Microsomal blank chromatogram for the rat.

CHROMATOGRAMS OF BENZO[*a*]PYRENE METABOLITE FORMATION

Representative chromatograms of hepatic microsomal B[*a*]P metabolite formation by control and βNF-treated avian species and the rat.

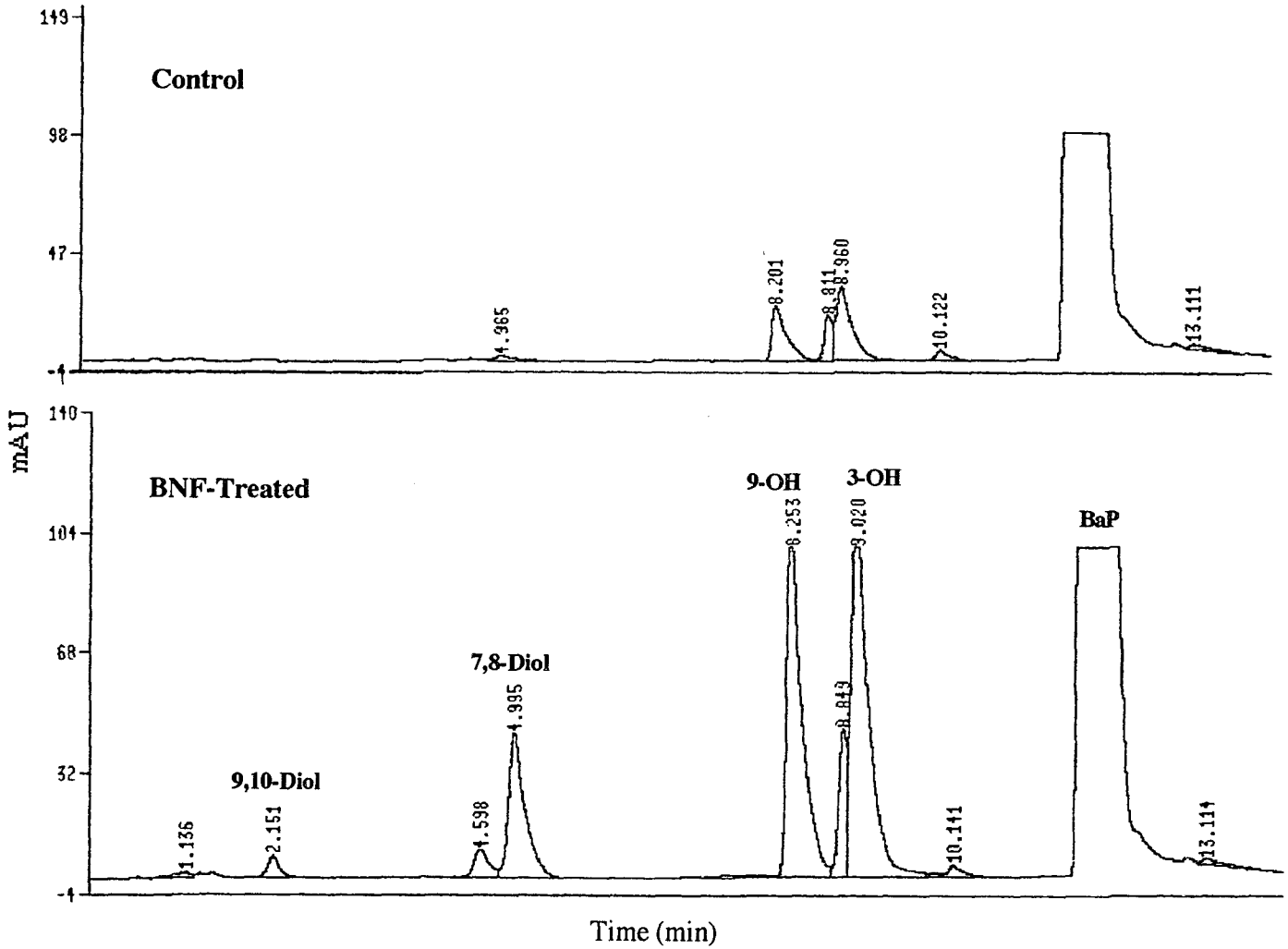


Figure A-31 Chromatograms of benzo[*a*]pyrene metabolite formation by chicken hepatic microsomes.

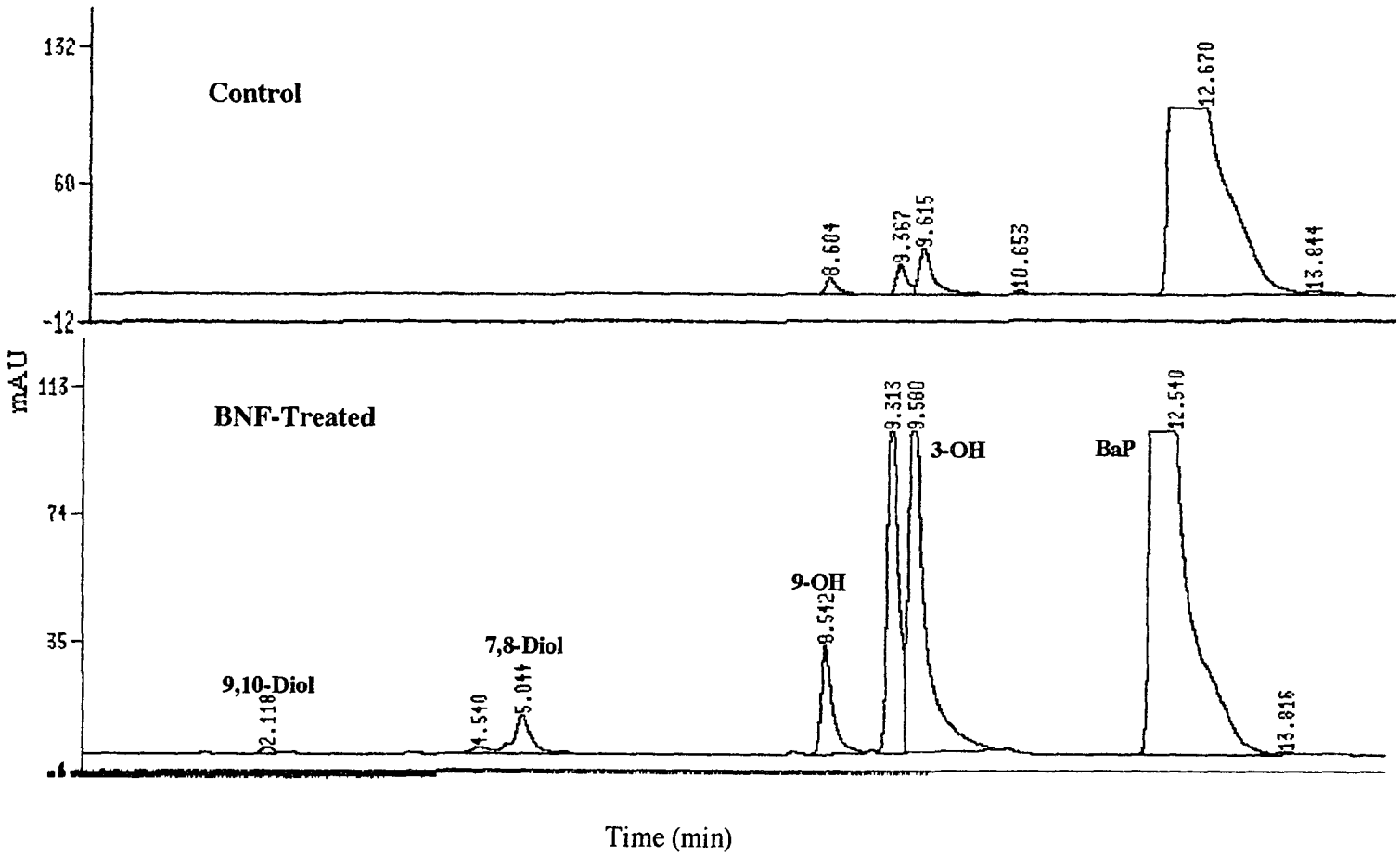


Figure A-32 Chromatograms of benzo[*a*]pyrene metabolite formation by Japanese quail hepatic microsomes.

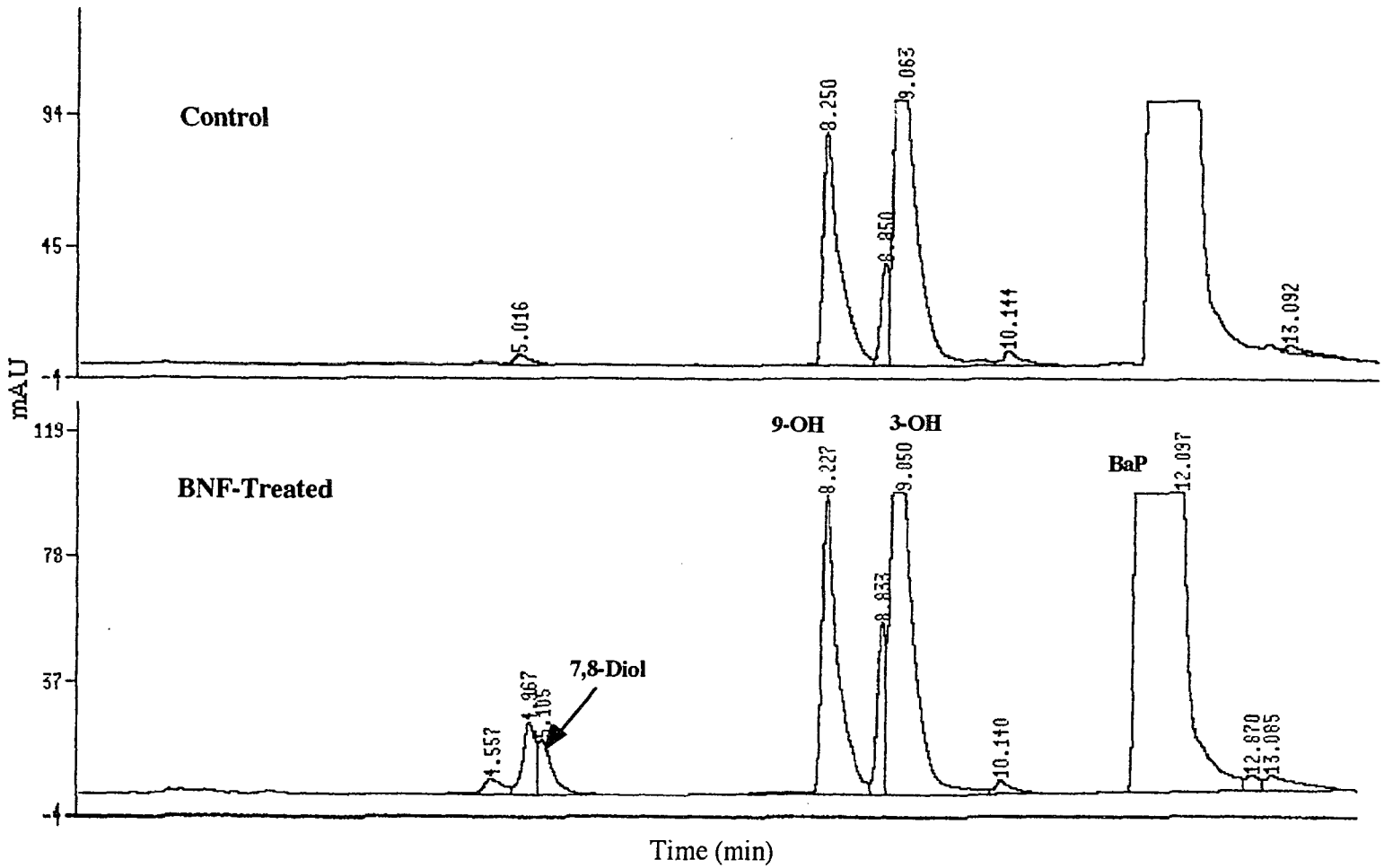


Figure A-33 Chromatograms of benzo[*a*]pyrene metabolite formation by turkey hepatic microsomes.

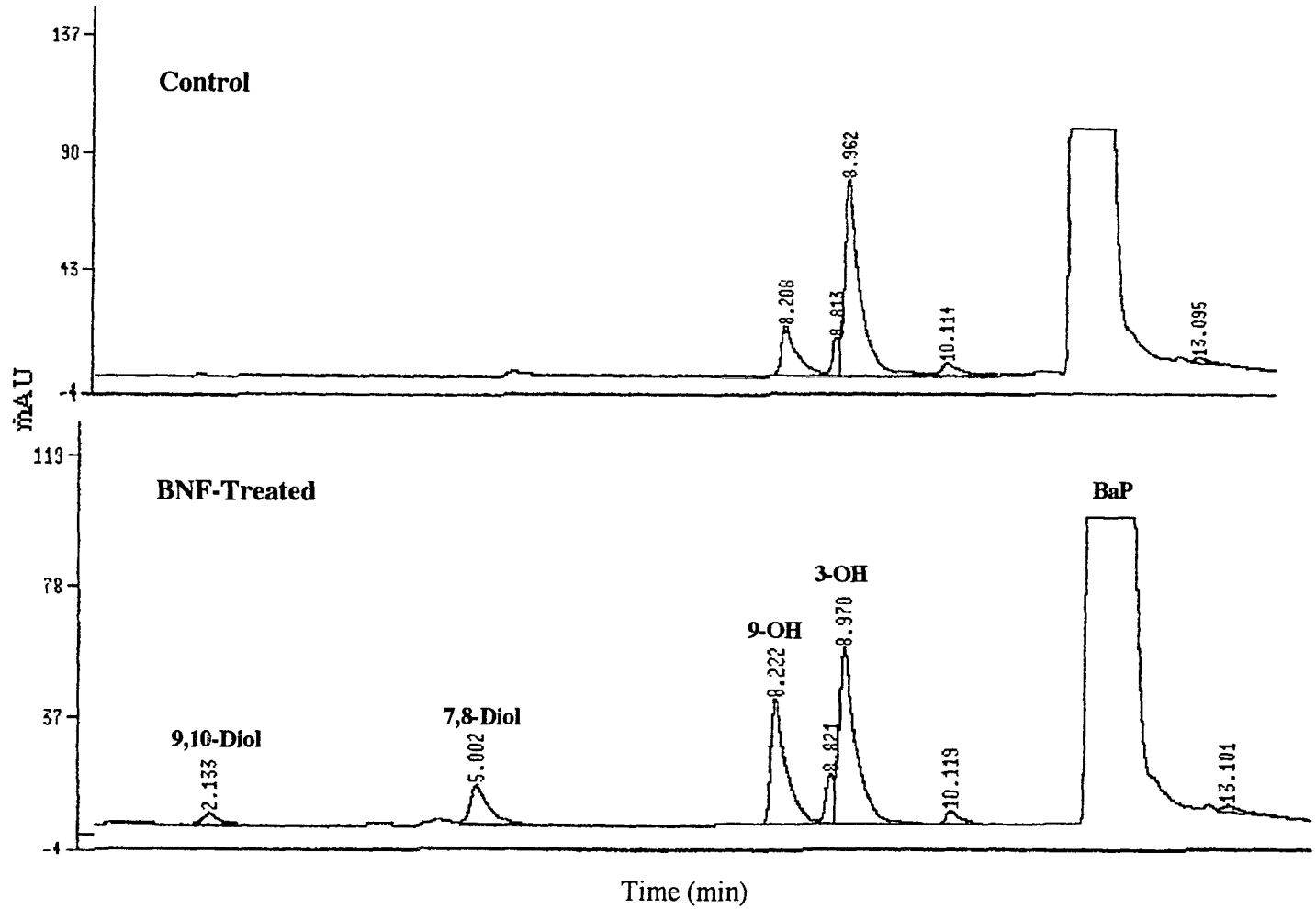


Figure A-34 Chromatograms of benzo[*a*]pyrene metabolite formation by goose hepatic microsomes.

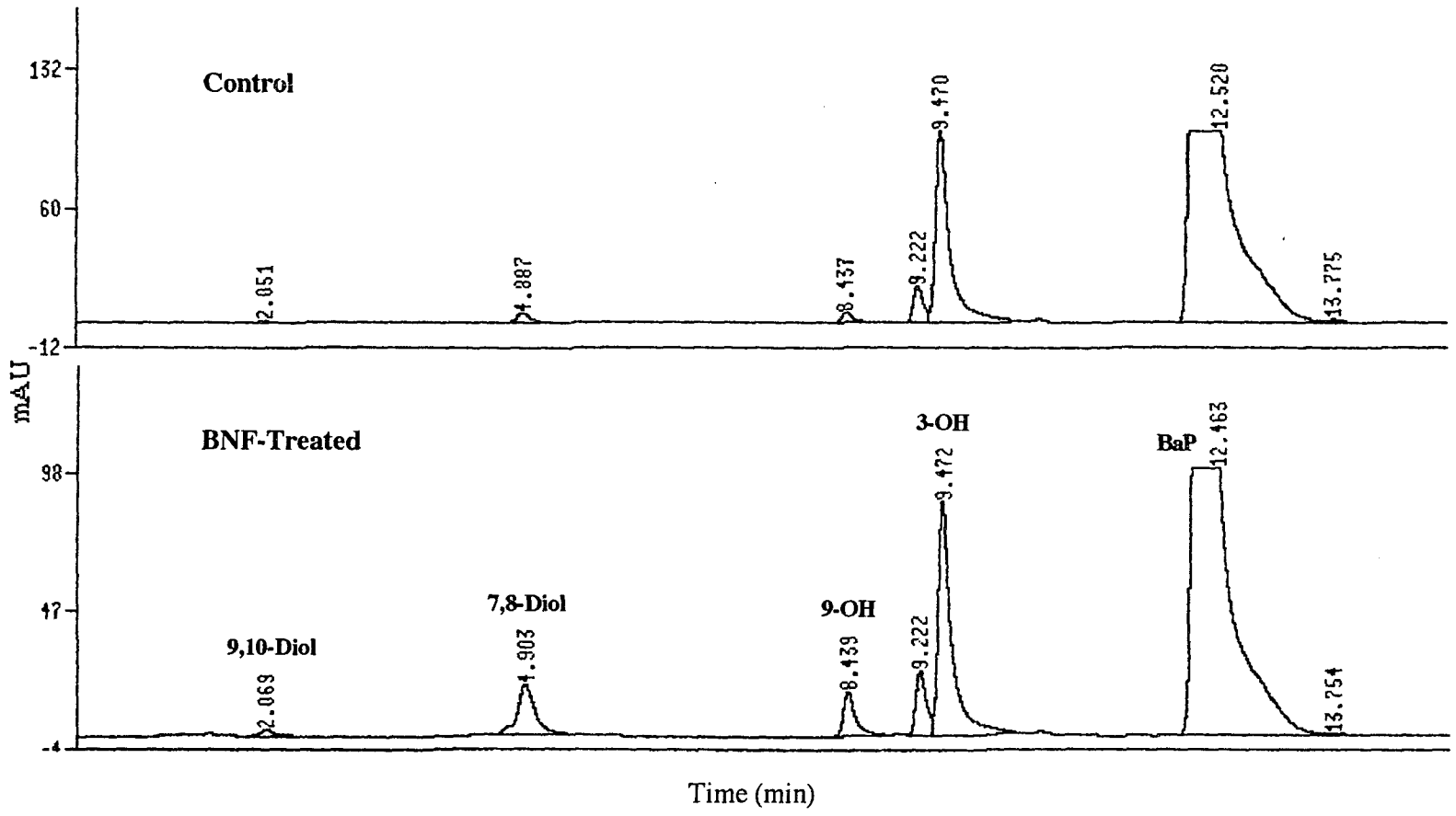


Figure A-35 Chromatograms of benzo[*a*]pyrene metabolite formation by Khaki Campbell duck hepatic microsomes.

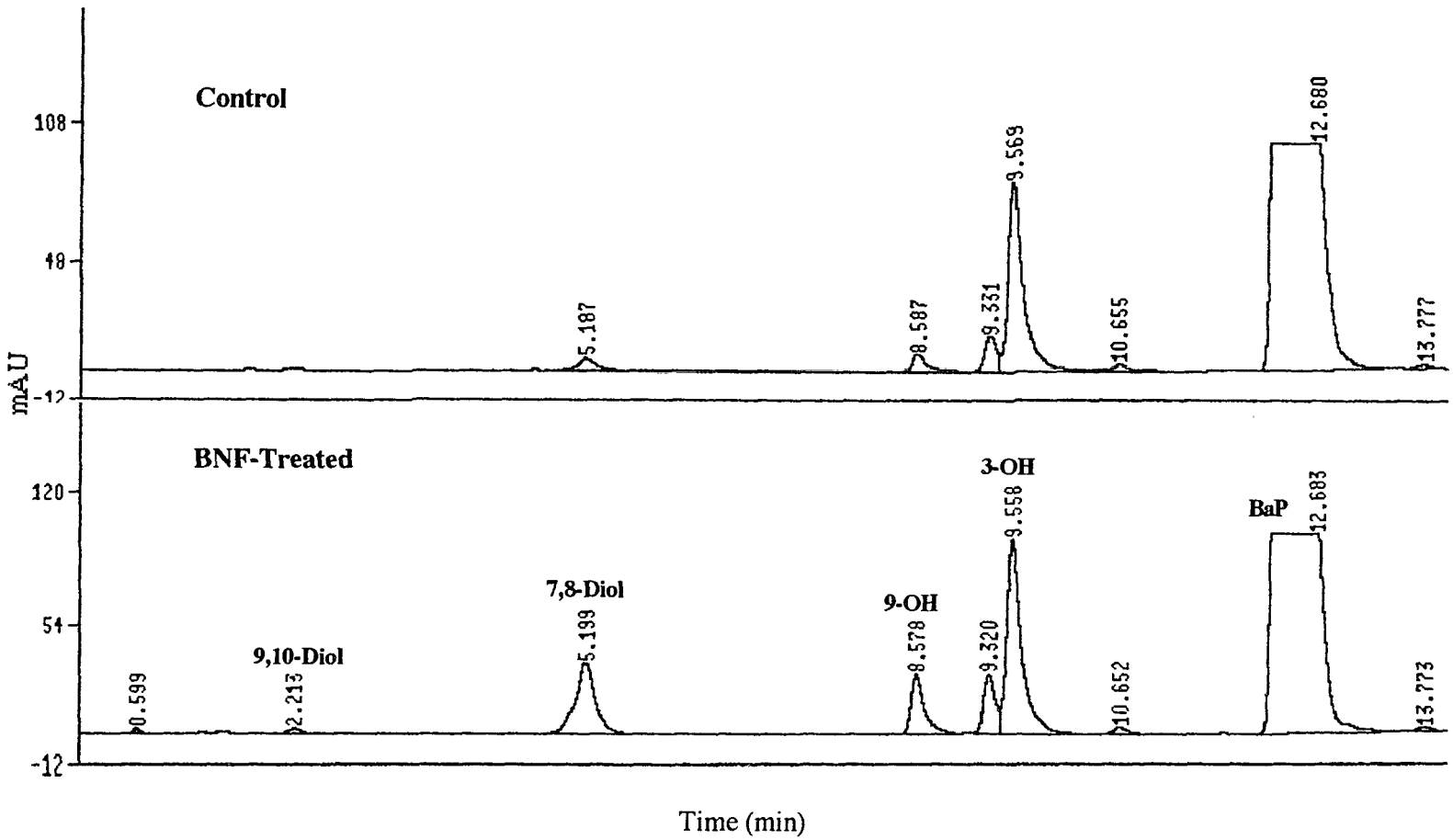


Figure A-36 Chromatograms of benzo[*a*]pyrene metabolite formation by Pekin duck hepatic microsomes.

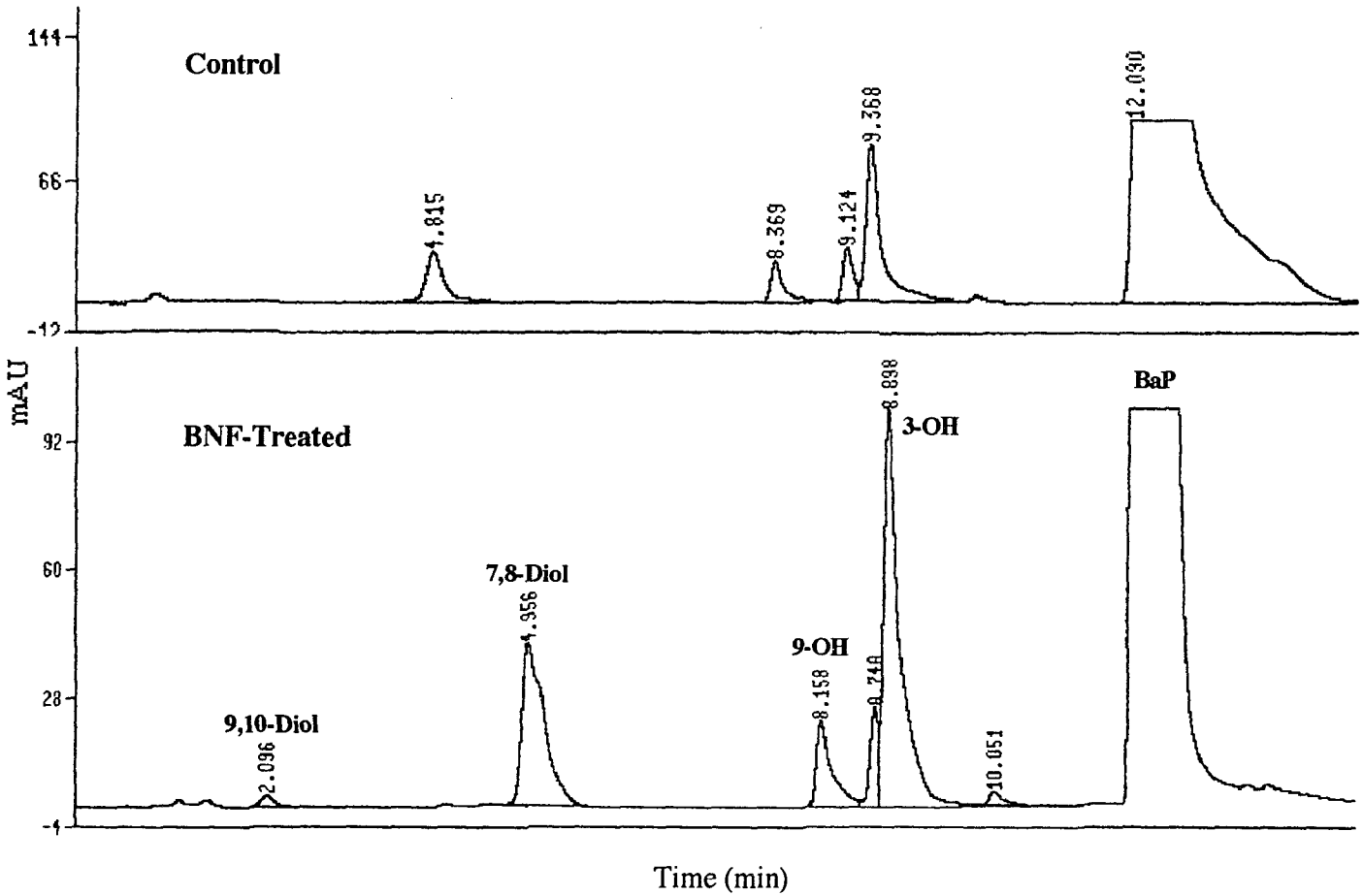


Figure A-37 Chromatograms of benzo[a]pyrene metabolite formation by Muscovy duck hepatic microsomes.

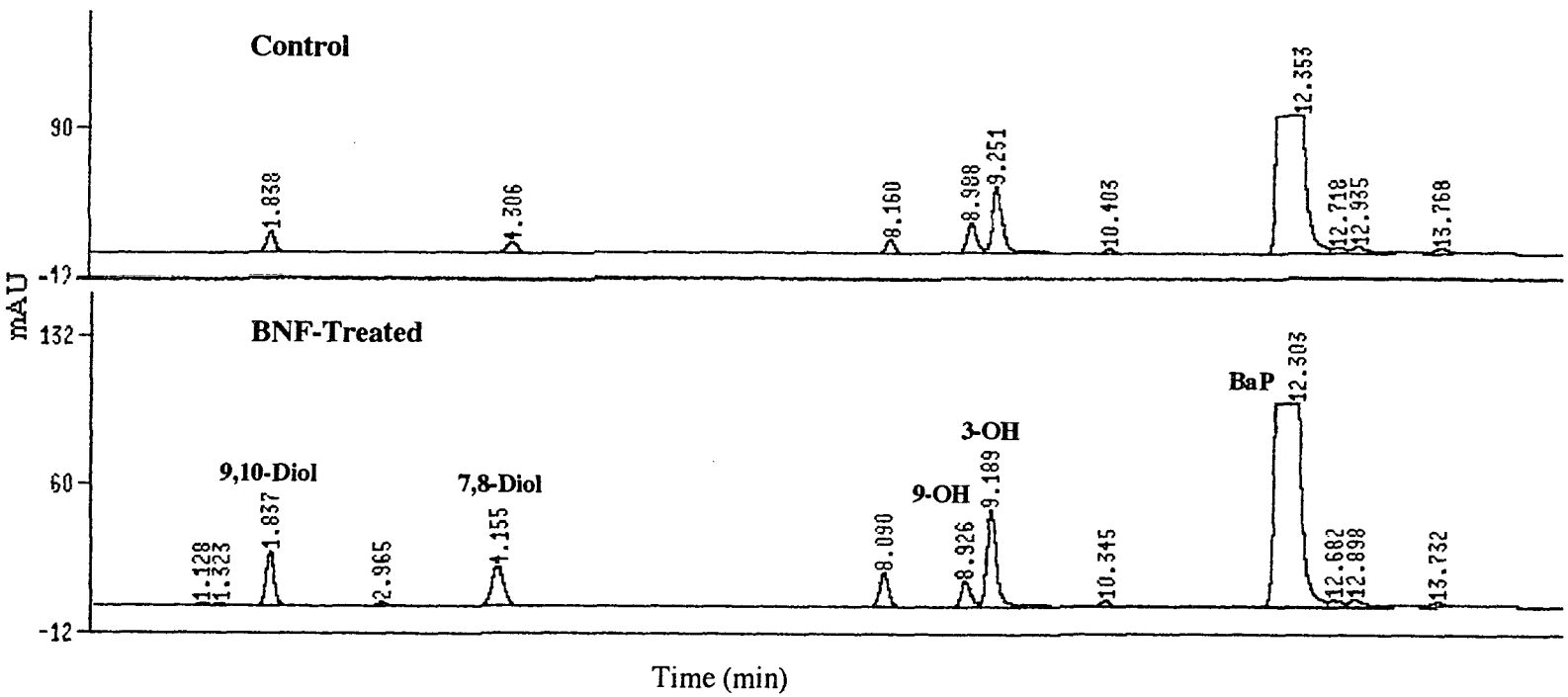


Figure A-38 Chromatograms of benzo[*a*]pyrene etabolite formation by rat hepatic microsomes.

VITA

June Lynn Sutherlin was born on November 6, 1959 in Port Sulfur, Louisiana. She graduated from River Oaks Academy, Belle Chasse, Louisiana, in May of 1977. Her undergraduate study was undertaken at the University of Southwestern Louisiana, Lafayette, Louisiana and was directed at meeting the Louisiana State University School of Veterinary Medicine admission requirements. After receiving a Doctor of Veterinary Medicine degree in May 1985, she was employed in private practice for 18 months. In the fall of 1987 she began graduate study at the Institute of Environmental Study, Louisiana State University, in the Masters program environmental toxicology option, at the Institute of Environmental Studies, Louisiana State University under the direction of Dr. Barbara Shane. In the summer of 1988, she transferred to the Ph.D. program at the School of Veterinary Medicine, Department of Veterinary Physiology, Toxicology and Pharmacology, Toxicology option. These studies were under the direction of Dr. Charles R. Short. From July 1989 until the present, she has been employed as a environmental consultant.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: June L. Sutherlin

Major Field: Veterinary Medical Sciences

Title of Dissertation: Comparative Hepatic Oxidative Biotransformation in Domestic Avian Species

Approved:

Co-Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

April 5, 1994